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TWO ATTEMPTS UPON IMPROVING AN INDUSTRIAL STRAIN OF *ASPERGILLUS ORYZAE* THROUGH SOMATIC RECOMBINATION AND POLYPLOIDIZATION

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As stated in the previous paper[1], the strain *Aspergillus oryzae* 51 has a parasexual life cycle instead of an orthodox sexual one, producing new types of segregants through somatic crossing-over and reduction of a heterozygous diploid. In addition, the authors have referred to the possible triploid and tetraploid strains which were produced from the combinations between a haploid and a diploid and between two diploid strains.

This paper is concerned with the experiments which were carried out, in parallel with those reported before, with the attempt to improve the strain through somatic recombination and polyploidization. With two biochemical features of the strain, kojic acid producibility and protease activity, not only qualitative change but also quantitative change were investigated in the following way.

METHODS AND MATERIALS

1. *Strains*

The strains reported in the previous paper were equipped with the following characters in addition.

Original strain: The original strain *Asp. oryzae* 51 was an industrial strain having strong protease activity comparable to any other industrial ones. Kojic acid producing ability was medium, 300–1000 γ/ml.

White leucineless weak producer: The white leucineless strain reported previously was a kojic acid weak producer, less than 300 γ/ml. This strain was obtained from a white leucineless medium producer by ultraviolet irradiation.

Yellow lysineless strong producer: The yellow lysineless strain described in the previous paper was a kojic acid strong producer, more than 3000 γ/ml. This strain was selected among plenty of yellow lysineless producers. Mutation from a medium producer to a strong producer might have occurred in

the course through which a green lysineless mutant was derived from the original strain

Diploid, triploid, and tetraploid strains: Of these, the history has already been given in the previous paper. The colonial and conidial types are to be given in Plates 1 and 2.

2. *Media*

Just the same as those having been described in the previous paper, except the special media which will be shown in the following sections.

3. *Somatic recombination and polyploidization*

The techniques employed for the production of heterocaryon, heterozygous diploid, triploid, tetraploid, and their segregants were described in detail in the previous paper. There was no alteration, since this paper is connected with only the additional characters which were omitted from the description at the time.

4. *Measurement of kojic acid*

A colorimetric method deviced by Sakaguchi[2] and Tatsumi[3] was employed. After cultivating tester strains in malt extract (Balling 8°), one ml of aliquot was withdrawn from separate cultures on different days. When the dilution to an appropriate concentration was completed, one ml of 0.5% ferric chloride solution and equal amount of 15% HCl solution (to eliminate analogous reactions) were added to 10 ml of the diluted solution. Comparing the resulting purple color with standard ones which were prepared from authentic crystal, the amount of kojic acid produced in the original cultures was recorded with mg in 100 ml. Precise measurement was performed with flask culture and with a colorimeter, but for screening purpose. a simplified method, with test tube culture and with the naked eye, was adopted.

5. *Measurement of protease activity*

Specially prepared wheat bran medium was employed for protease production. After charging 100 ml Erlenmyer flasks with 5 g of wheat bran and 4 ml of water, and exceptionally with 12 mg of *L* leucine or *L* lysine in some of the flasks, they were sterilized at 120° for 10 minutes. Inoculation was made from the cultures which were pre-incubated for 2–5 days on wheat bran medium. The leucine- or lysine- added media served for leucineless or lysineless strains resp., because they did not show good growth on plain wheat bran medium unless the medium was supplemented with either of the amino acids. The amount, 12 mg per each flask, was determined preliminary to be the best for growth and protease production.

After three days when protease activity reached the maximum, seven flasks per each strain were harvested. First, they were shaken vigorously with the hands, and then were placed at room temperature for 10–15 hours with the addition of 50 ml of 1% NaCl solution and 0.5 ml of toluol. It was confirmed that the shift of protease from wheat bran to filtrate was com-

plete through this process and the following washing. The filtrate was filled up to 500 ml with washing water.

Casein solution, the substrate for protease measurement, was prepared as follows: Mixing 2 g of milk casein, 20 ml of water, and 2 ml of 1 N NaOH solution in a flask, the resulting past was heated on a water bath. When the added casein had completely dissolved, the mixture was neutralized with 1 N HCl solution. After then, the solution was filled up to 100 ml with 45 ml of M/15 phosphate buffer and distilled water.

For enzymation, one ml of enzyme solution having been warmed up in advance at 35° for 3 minutes in separate test tubes was charged with one ml of casein solution which had also been warmed up just as the enzyme solution. After 10 minutes, during which the test tubes were kept at 30° in a water bath, 2 ml of trichlor acetic acid was added from a pipette. Then, after letting the test tubes stand again for 20 minutes at 30°, the solution was filtered through small funnels.

Measurement was carried out as follows: One ml of the filtrate mentioned above was reacted with one ml of Folin's reagent[4] which had been diluted in advance to one fifth of the standard concentration. When another 20 minutes passed, the resulting color was measured with a colorimeter. Correcting the observed values by blank tests, enzyme activity was figured out with the reading on a colorimeter, because the readings thus obtained were proportional to the enzyme activity in the range where the measurement was performed.

RESULTS

1. *Improvement of kojic acid producibility through somatic recombination*

The authors adopted kojic acid producibility of the strain as a suitable biochemical marker for breeding purpose, for the reason that kojic acid is a special fermentation product of *Aspergillus oryzae-flavus* group of fungi, amounting, at the best, to more than fifty percent of the consumed sugar, and being able to be measured by a simple colorimetric method.

(1) Heterocaryons

Twelve heterocaryons, arising from different plates, were produced between a white leucineless kojic acid weak producer and a yellow lysineless strong producer by the method described before. The kind of strains which had green (heterocaryon), white, and yellow (components) conidia were purified again on minimal medium. When plenty of small pellets of mycelia appeared in liquid minimal medium, they were picked up with a needle and washed up with sterile water in order to prepare such inocula which contain heterocaryon only. In this medium only heterocaryotic conidia could germinate, while the component types, even if they might co-exist, could not.

Cultivation was carried out in 100 ml Erlenmyer flasks containing

20 ml of malt extract, and the amount of kojic acid produced was measured after 6, 8 and 10 days with a colorimeter. The figures in Table 1, corresponding to the maximal value of each culture, suggest that kojic acid production might have been fixed in dominance over non-production in the existing condition, although the final composition of two types of nuclei, the strong producer and the weak producer, has not yet been examined.

Table 1. Production of Kojic Acid by the Heterocaryons and the Heterozygous Diploids which were Produced between a White Leucineless Weak Producer and a Yellow Lysineless Strong Producer*.

	Kojic acid produced (γ/ml)				$\overline{m}=\sum x/N$	$s=\sqrt{\sum d^2/N-1}$
Heterocaryons (12 strains)	3,000	5,250	4,000	4,500	4,158	711
	4,500	4,500	3,750	3,400		
	4,500	3,750	5,250	3,500		
Heterozygous diploids (11 strains)	600	750	350	875	566	178
	750	625	450	400		
	400	400	625			

Cultivation was carried out in parallel, using two flasks per each strain. The values the in table indicate the maximal amount of kojic acid produced, corresponding to about 10% of the consumed sugar in the case of heterocaryons.
* As to the kojic acid production by the parental strains, see Table 3.

(2) Heterozygous diploids

In contrast to the heterocaryons which produced kojic acid in the same order as that of the component strain (yellow lysineless producer), all of the heterozygous diploids having been derived from the above mentioned heterocaryons by means of ultraviolet irradiation (regarding the technique, see the previous paper[1]) were proved to be weak producer as seen in Table 1. Of the inconstancy found between the heterocaryons and the heterozygous diploids, the authors wish to discuss in a later chapter.

(3) Segregants recovered from a heterozygous diploid

As demonstrated in the previous paper, 10 kinds of phenotypically different segregants were recovered from one of the above mentioned heterozygous diploids. Kojic acid producibility was tested without knowing whether each of the strains was a haploid or a diploid.

The first screening was made with test tube culture, and out of the strains tested, strong producers and weak producers were left for additional tests. Table 2 is the summarized data thus obtained, showing the distribution of kojic acid producibility among 206 segregants tested. It was unavoidable that the shift from strong producer to medium or weak producer and *vice versa* had occasionally taken place during the time through which the first and the second tests were performed.

In spite of the instability experienced with some segregants, at least

Table 2. Distribution of Kojic Acid Producibility among 206 Offsprings which Segregated from a Heterozygous Diploid*.

Phenotypes	No. of strains produced kojic acid in amount of (γ/ml)			Total
	<300	300–1.000	$1.000<$	
White leucineless	24	0	1	25
White lysineless	10	0	3	13
White leu-lys-less	6	3	4	13
White prototroph	8	0	1	9
Yellow leucineless	0	0	0	0
Yellow lysineless	6	0	77	83
Yellow leu-lys-less	0	0	0	0
Yellow prototroph	17	2	5	24
Green leucineless	4	5	3	12
Green lysineless	0	2	1	3
Green leu-lys-less	2	0	1	3
Green prototroph	22	2	0	24

* As to the kojic acid production by the parental strains, see Table 3.

Table 3. Production of Kojic Acid by the Parental Strains and Some Representative Segregant Strains.

	Strains	Kojic acid produced (γ/ml)					$\bar{m}$	s
Parents	White leucineless (No. 23)	300	240	300	300	300	288	26
	Yellow lysineless (No. 22)	4,000	5,000	4,500	4,500	3,750	4,350***	487
Segregants	No. 109 (Yellow lysineless)	3,000	4,500	4,000	5,000	5,000	4,300	837
	No. 160 (Yellow lysineless)	4,500	5,250	3,750	3,750	4,500	4,350	627
	No. 212 (Yellow lysineless)	3,500	4,500	4,000	3,250	3,750	3,800	481
	No. 215 (Yellow lysineless)	4,500	5,250	5,250	4,500	6,000	5,100	628
	No. 216* (White prototroph)	5,250	(10,000)**	6,000	6,000	6,400	5,912***	480

Each strain was cultivated with five flasks.

* This strain was proved to be diploid.

** This value was omitted from the calculation.

*** The difference between No. 22 and No. 216 strains was calculated to be statistically significant. $t=4.822$, $t_0=2.365$, $n=7$, $Pr\{t>t_0\}<0.05$. Moreover, there was no delay in kojic acid production.

Table 4. Comparison of Protease Activity found in three Haploid, three Diploid, one Triploid, and one Tetraploid Strains.

Strains	Protease activity (readings on a colorimeter)							$\bar{m}$	s
No. 51 (haploid) Original strain	32	24	25	26	25	25	20	25.3	3.55
No. 23 (haploid) White leucineless	21	22	26	23	26	20	21	22.7	2.53
No. 22 (haploid) Yellow lysineless	37	27	32	40	30	31	35	33.1*	4.41
No. 7 (diploid) Green prototroph	50	51	41	45	43	43	47	45.7*	3.79
No. 37 (diploid) Green leucineless	40	30	38	24	28	30	43	33.3	7.05
No. 166 (diploid) Green lysineless	52	49	46	38	40	40	51	45.1*	5.79
No. 3 (triploid) Green prototroph	37	23	20	18	23	15	40	25.1	10.28
No. 1. (tetraploid) Green prototroph	59	47	50	46	55	43	56	50.9*	5.93

Cultivation was carried out for three days.
* The differences found between No. 7 and No. 22, between No. 166 and No. 22, and between No. 1 and No. 22, were calculated to be statistically significant.

one kojic acid strongly producing white leucineless segregant and three kojic acid weakly producing yellow lysineless segregants were proved to be still keeping the character even after six months. Moreover, the fact that no further segregation was observed in these strains suggested they might be haploid. Since the original white leucineless strain was a weak producer and the original yellow lysineless strain was a strong producer, it might be reasonable to say here that kojic acid producing ability had transmitted, through diploidization and somatic recombination, from a strong producer to a weak producer and *vice versa*. This transmission could be interpreted with the idea which the authors have adopted for the transmission of amino acid requirement. Neverthless, the authors know well that additional factors must be taken into account. A brief discussion will be made later.

With reference to the data obtained in the screening tests, five strong producers were selected for the championship. Cultivation was made in parallel with parental strains. As seen in Table 3, No. 216, white prototrophic segregant, produced about fourty percent larger amount of kojic acid than the parental producer, yellow lysineless, but no significant increase in kojic acid producibility was observed in other segregants.

2. *Improvement of protease activity through polyploidization*

In spite of the fact that the authors had produced no more positive evidence of the ploidy than those published in the pervious paper, the protease activity of the possible triploid and tetraploid strains were examined

along with that of haploid and diploid strains. Among them, prototrophic strains were cultivated on plain wheat bran medium, while auxotrophic strains such as leucineless and lysineless ones were cultivated on the leucine- or lysine-added media. The technique adopted for the measurement has already been given in the preceding chapter.

The figures in Table 4 show the readings on a colorimeter which are proportional to protease activity, and the differences found between two diploid strains (No. 7, No. 166) and a haploid strain (No. 22), and between a tetraploid strain (No. 1) and a haploid strain (No. 22) were proved to be statistically significant.

Table 5. A Survey on the Relationship between Protease Activity and its Genetic Background.

Strains	Genotypes	*leu* : *lys*	*w* : *y*	$\bar{m}$ (from Table 4)
No. 51	*W Y LEU LYS*	0 : 0	0 : 0	25.3
No. 23	*w Y leu LYS*	1 : 0	1 : 0	22.7
No. 22	*W y LEU lys*	0 : 1	0 : 1	33.1
No. 7	$\frac{w\ Y\ leu\ LYS}{W\ y\ LEU\ lys}$	1 : 1	1 : 1	45.7
No. 37	$\frac{w\ Y\ leu\ LYS}{W\ y\ leu\ lys}$	2 : 1	1 : 1	33.3
No. 166	$\frac{w\ Y\ leu\ lys}{W\ y\ LEU\ lys}$	1 : 2	1 : 1	45.1
No. 3	$\frac{w\ Y\ leu\ LYS}{W\ y\ leu\ lys}$ $\frac{}{W\ y\ LEU\ lys}$	2 : 2	1 : 2	25.1
No. 1	$\frac{w\ Y\ leu\ LYS}{W\ y\ leu\ lys}$ $\frac{w\ Y\ leu\ lys}{W\ y\ LEU\ lys}$	3 : 3	2 : 2	50.9

Later on, the improved tetraploid strain was demonstrated able to rank itself, in its protease activity, with any other first grade industrial strains.

DISCUSSION

In the preceding chapter, the authors have presented two positive data supporting the view that the process of somatic recombination and polyploidization in fungi might be available for breeding purpose. Neverthless, the authors wish to add some comments to avoid misunderstanding.

First, the authors are afraid that the number of experiments is too small and the results so far obtained are not so significant as to be able to draw a conclusion. As to the reason why the breeding effect was not so significant, the authors think that it is due to the combination of strains with

which the crosses were carried out. All of the crosses appearing in this paper were performed between two mutant strains which were derived from the same source, a combination of which may not be expected significant effect because of their analogous genetic background. For the reason, the authors have already turned to the attempt to produce a hybrid between *Asp. oryzae* and *Asp. sojae*. The first paper will appear before long.

Regarding the kojic acid producibility of the strain, the authors think it is controlled by nuclear genes. The heterocaryon produced between a strong producer and a weak producer segregated strong producers and weak producers when the heterocaryon produced conidia again. Moreover, all of the heterocaryons so far produced between two non- or weak producers were proved to be non- or weak producers, suggesting that, in these strains, the same locus responsible for kojic acid production might have been damaged in the same way. In this connection, it is worth while noting that similar phenomenon has been reported by Bonner[5] and Sermonti[6] with *Penicillium*. According to them, all of the trials which were intended to creat a producer heterocaryon from two penicillin non or weak producers had resulted in failure.*

In spite of the absoluteness of gene control upon kojic acid production, the quantitative aspect of this acid production seems to be influenced by other factors. It is possible to think of gene interaction as Strauss[7] has suggested in his paper dealing with such enzymes which are concerned with sugar metabolism. Of this, the authors have no additional evidence except for the fact that kojic acid producibility had distributed continuously among segregants. Accordingly, one possible explanation for the transmission of kojic acid producibility from a strong producer to a weak producer and *vice versa* might be that the production and non-production were the reflection of the new genetic background which resulted from somatic recombination. But still, if it does them justice to make a speculation on the same ground as that upon which the authors have discussed the transmission of amino acid requirement, the gene responsible for kojic acid production might locate on the same chromosome as lysine gene does.

Of the reason why kojic acid producibility was fixed in dominance in the heterocaryons and be fixed in recessive in the heterozygous diploids, the authors have to thank Dr. Sermonti for his personal communication, in which he kindly suggested that the kind of heterozygous diploids which were produced between a strong penicillin producer and a weak producer being derived from the former had always behaved as strong producers, that is, mutation occurred to recessive direction. In the author's case, the strains, strong producer and weak producer, were derived separately from the original strain which produced kojic acid in the medium amount. Therefore, the authors wish to refrain from making universal remark that kojic

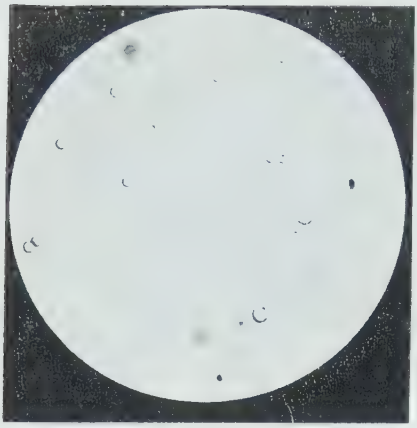
* Recently, an exception was reported by G. Sermonti (*J. gen. Microbid.*, **15**, 599 (1956)).

PLATE 1. Colonial types

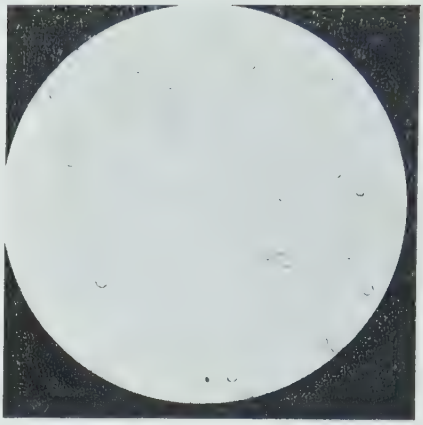
Upper left —No. 23 (haploid)
Center left —No. 22 (haploid)
Lower left —Heterocaryon ($^{22}/_{23}$)

Upper right —No. 7 (diploid)
Center right —No. 3 (triploid)
Lower right —No. 1 (tetraploid)

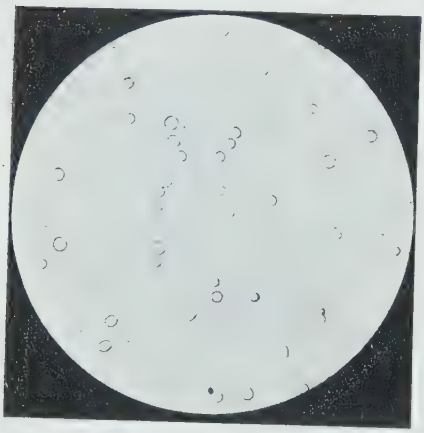
PLATE 2. Conidial types



No. 23 (hadloid)

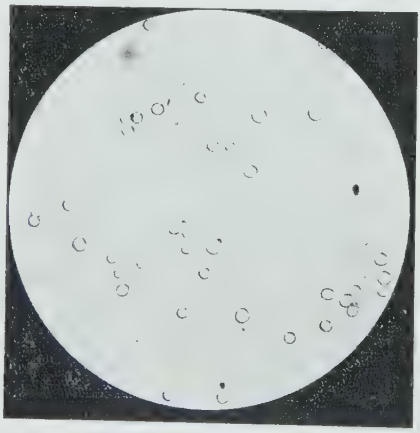


No. 7 (diploid)



No. 22 (haploid)

No. 3 (triploid)



Heterocaryon (22/23)

No. 1 (tetraploid)

acid non-production is dominant over production. On the other hand, production of kojic acid by the heterocaryons will be explained as follows; the strong producer nuclei had taken a selective advantage in reproduction over the weak producer nuclei in the existing condition.

In contract to the lot of comments made on kojic acid production, the authors have not so much to say about protease production. As a reference, the authors wish to show a table (Table 5) indicating the relationship between protease production and its genetic background.

SUMMARY

1. Improvement of kojic acid producibility through somatic recombination was attempted with the following results:

(1) One white leucineless strong producer and three yellow lysineless weak producers were recovered from a heterozygous diploid which was produced between a white leucineless weak producer and a yellow lysineless strong producer.

(2) One of the segregants obtained from the above mentioned diploid strain produced about fourty percent larger amount of kojic acid than the parental producer, yellow lysineless strain.

(3) In general, the breeding effect through this process did not turn out to be so significant as expected.

2. Protease activity of three haploid, three diploid, one triploid, and one tetraploid strains were compared by wheat bran koji method, with the result that the protease activity of two diploid and one tetraploid strains was significantly higher than any of the haploid ones.

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TAXONOMIC STUDIES ON THE HIOCHI-BACTERIA, SPECIFIC SAPROPHYTES OF SAKE
I. ISOLATION AND GROUPING OF BACTERIAL STRAINS

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Although more than 15 per cent of alcohol is contained in the Japanese saké, it is often attacked by specific bacteria, which render saké turbid and smell and taste abominable. This phenomenon and the organisms have been called 'hiochi' and 'hiochi-bacteria' respectively. The organisms were first observed by ATKINSON[1] in 1881, their natures were studied successively by K. OTANI[2], TORII[3], TAKAHASHI[4], YAMASAKI[5], and Y. OTANI[6]. But, owing to the difficulties of culture and maintenance, in spite of the efforts of these excellent seniors, hiochi-bacteria have not yet been clearly known.

In relation with the difficulty of culture, nutritional demands of the hiochi-bacteria were investigated by KURONO[7], YAMASAKI[8] and Y. OTANI. These researches are very interest from historical point of view, for the knowledges in this field have recently been markedly increased by the development of bioassay. Researches attempting to prevent saké from hiochi by eliminating the growth factors or adding their antagonic substances have recently become prevalent[9][10][11]. It may be very necessary to get a clearer knowledge about the natures of the organisms.

As the general conclusions from results of those senior investigators, hiochi-bacteria are outlined as follows. They are as a rule GRAM-positive, non-spore-forming, non-motile, catalase negative, facultatively anaerobic, rod-shaped bacteria, having very complex nutritive requirements. They require various substances such as amino acids, vitamins, purines and pyrimidines, and perhaps certain peptides. They can grow in ethanol-containing media, the concentration of which amounts as high as 12–16%, and in acidic media such as pH 4.0 to 5.5.

TAKAHASHI[12] divided the hiochi-bacteria into two groups, namely "hiochi-lactobacilli" and "true hiochi-bacilli." The former can grow not only in saké but also in ordinary culture media such as glucose-bouillon, glucose yeast water, etc., while the latter cannot grow in almost any natural nutritive media other than saké.

At present, it is generally considered that hiochi-bacteria come within the category of lactic acid bacteria. In fact, YAMASAKI showed that there are two

types of hiochi-bacteria, one of which forms only lactic acid from sugar and the other type produces considerable amount of CO_2 besides lactic acid.

Takahashi[12] proposed the name *Bacillus saprogenes Saké* to his "true hiochi-bacillus," and claimed that his "hiochi-lactobacilli" were varieties relating to *Bacillus panis fermentati*, *B. lactis acidi*, *B. aderholdi*, *B. wortmannii*, *B. delbrückii*, respectively. Y. Otani, on the other hand, concluded that hiochi-bacteria can be classified to *Lactobacillus hyochi*, *L. fermentans*, *L. alcoholophilus* and *L. saprogenes* depending on the differences in fermentable sugars, alcohol-resistance, etc. For classifying lactic acid bacteria, type of fermentation either homo or hetero, and optical form of lactic acid must be determined besides ordinary morphorogical and physiological characteristics. The purpose of this research is to construct a clearer idea upon the nature of hiochi-bacteria from the view point of lactic acid bacteriology.

EXPERIMENTAL

Strains: Newly isolated 53 strains from 52 samples of spoiled saké were added to the 11 strains which had been isolated in 1950 by one of the present authors (Kaneko).

Method of Isolation: a) *Preliminary enrichment*:—One to five drops of spoiled saké were introduced in 5 to 8 ml. of Yamasaki's liver piece-saké medium[13] and incubated at 30°C. Growth could be observed after 5 to 20 days.

b) *Isolation*:—Liver infusion-saké agar.....400 g of fresh liver was boiled in water and the infusion was made up to 500 ml, mixed with 3 to 4 volumes of saké, solidified with 1% agar, and sterilized for 10 minutes in an autoclave under 10 psi..... in Burri-tube was used for pure culture of the bacteria. As a rule, fist or pinhead colonies develop after 5 to 15 days' incubation. The organism is picked out from a colony and inoculated to a saké medium containing a cubical piece or extract of liver. This procedure is repeated if necessary.

c) *Maintenance of the strains*:—Stab culture in liver extract-saké-agar medium is used for the maintenance of hiochi-bacteria. The organisms grow along the stab-canals after 3 to 10 days at 30°C. After recongnizing their growth, they are preserved in an ice box, and transplanted monthly.

General Characteristics: Among 64 strains, only several members could grow in ordinary media such as glucose-yeast extract or glucose-bouillon, while all strains could grow in saké containing liver or its extract, or in a semi-synthetic medium containing 'hiochic acid' which had been discovered by G. Tamura[14] as a component of saké and is an essential growth factor for hiochi-bacteria.

Liquid culture:—As a rule, the growth of hiochi-bacteria is slow, they begin to grow only after 3 to 7 days. But, strains which are comparatively

simple in nutritional demand can grow quite well in one day or two. No strain can grow on the surface. Turbidity of the liquid medium is as a rule not so remarkable, but reveals silky lustre by shaking. The cells sediment gradually, and in older culture the turbidity is almost faded away and there remains only a compact sediment.

Solid culture:—When stabbed in liver extract-saké-agar medium, all strains grow uniformly along the stab-canal in filiform or papillate, and never reveal remarkable surface or bottom growths. Therefore, hiochi-bacteria are concluded to be microaerophilic. Colonies formed in deep places are generally of fist-shape having 1 mm diameter, white and opaque, and strongly cohasive. As a rule, almost no growth can be detected in streak culture.

Morphology and Physiology:—Morphological and ordinary physiological characters are examined on the organisms cultured for 5 and 12 days in the liver extract saké. All strains, except one, are rod-form having 0.7–0.8 μ breadth. The length of them varies by strains, namely, from short rods of 1.5–2 μ to ordinary rods of 3–6 μ, however, in some strains longer cells of 10–20 μ or sometimes more than 30 μ can be seen. The cells exist singly, in pairs or in chains.

Without exception, they are GRAM-positive, non-motile, sporeless, catalase negative, and cannot reduce nitrate to nitrite. Indole formation can also never be detected.

Milk culture:—Milk was acidified and coagulated strongly by strain H-76 and weakly by H-26. However, these strains are rather exceptional and most of the hiochi-bacteria can never grow in milk even when the pH is adjusted to 5.5.

Gas formation:—After inoculation of a strain to the liver extract-saké medium, sterilized 2% agar solution is carefully poured and solidified over the medium. If the strain is a gas-former, fine bubbles may accumulate between the solid and liquid layers. Among 64 strains, 49 strains including one spherical strain were determined to be gas-formers.

From these general experimental results, it may be concluded that the factors available for classification of the hiochi-bacteria are reduced to nutritive requirement and gas formation.

Classification of Hiochi-Bacteria by the Fermentation Formulas: Dr. YAMASAKI concluded that the hiochi-bacteria can briefly be classified into two groups, one of which forms lactic acid almost quantitatively from sugars, while the other group produces considerable amount of CO_2 besides lactic acid. The yield of lactic acid in the latter group is recorded as about 50% of glucose. From the view point of lactic acid fermentation, these phenomena correspond to homo- and hetero-lactic fermentations respectively. We have observed that our strains can also be divided depending on the gas production. We cultured each strain in liver extract-koji extract medium and the medium was analysed[15] after the fermentation was sufficiently subsided. The results are summarized in Table 1.

Table 1. Fermentation Products from Glucose.
Culture: 150 ml., 30 C, 7–10 days.

Representative strains	Number of analogous strains	Glucose consumed g	Products				
			Ethanol g	Acetic acid g	Lactic acid Amount g	Lactic acid Optical form	CO_2
H 1	42	2.63	0.54	0.22	1.16	DL	+
H 46	2	2.55	0.55	0.19	1.27	L	+
H 50	4	3.32	0.55	0.21	1.35	DL	+
H 14	6	2.42	0.04	0.08	2.26	D	–
H 17	5	1.40	0.03	0.02	1.47	D+DL	–
H 7	3	1.84	0.02	0.03	1.82	L+DL	–
H 26	1	1.65	0.05	0.08	1.36	DL	–
H 35–1	1	1.56	0.36	0.16	0.78	D	+

L-lactic acid: dextrorotatory, D-acid: laevorotatory.

As is seen in the table, all of our strains can be considered to come in the category of lactic acid bacteria. The exceptionally obtained spherical strain H-35-1 may belong to the genus Leuconostoc, as it performs hetero-lactic fermentation. Forty eight strains, namely over 80% of total, are also hetero-fermenters and the remaining 15 strains are homo-fermenters.....The optical properties of lactic acid will be discussed later in the part II of this series of research.

Classification of Hiochi-Bacteria by Nutritional Requirements: The experimental data from the view point of nutritional requirements can be summarized in Table 2.

Table 2. Nutritional Requirements of Hiochi-Bacteria.

	Representative strain	Number of analogous strains	ml N/10 NaOH per 4 ml. medium:					Dependency on	
			B	B+T	B+L	B+L+T	B+L+S	Factor L	Factor T
Hetero-fermenters	H 4	44	0.10	0.13	0.20	4.79	4.45	Essential	Essential
	H 34	4	2.32	2.05	3.97	4.13	4.45	Promotive	Independent
Homo-fermenters	H 42	7	0.21	0.11	0.12	5.74	5.54	Essential	Essential
	H 13	3	0.14	0.11	2.91	7.68	8.11	Essential	Strongly promotive
	H 38	1	0.10	0 18	1.37	1.65	3.60	Essential	Weakly promotive
	H 68	4	2.98	3.31	7.89	8.95	8.41	Promotive	Independent
Spherical strain	H 35–1	1	0	0.18	1.14	2.23		Essential	Promotive

We chose the following culture media for comparison of nutritional requirements:

Medium I or basal medium, (B):—"Basal Medium A" for hiochi-bacteria used by TAMURA[16].

Medium II, (B+T):—Basal Medium+Factor T (hiochic acid concentrate 10 mg per liter).

Medium III, (B+L): Casein hydrolysate and three amino acids of basal medium were substituted by 5 g of 'Polypeptone TAKEDA' and liver autolysate from 20 g fresh liver. This medium may be considered to be nearly equal to the basal medium supplemented with some peptides (we call them Factor L) originating from Polypeptone and/or liver.

Medium IV, (B+L+T):—Hiochic acid concentrate is supplemented further to the Medium III.

Medium V, (B+L+S):—Medium III+100 ml saké (S).

Most of the heterofermenting rods (44 strains) can not grow either in the basal medium or the media which were supplemented with Fator T or L, but they can grow well in the basal medium supplemented simultaneously with these two factors. They can grow equally well when the Factor T of the medium IV is replaced by saké.

Remaining four strains of the heterofermenters can grow fairly well in the basal medium. Factor T has no effect for these strains, whereas Factor L has a distinct stimulatory effect.

The heterofermenters can thus be divided into two groups by whether they demand the Factor T, i. e. hiochic acid, as a growth factor.

It is rather complicated in the case of homofermenters. Seven strains represented by H-42, demand both Factors, T and L, like the majority of the heterofermenters, and the growth of H-68 and other two strains is, like the minor group of heterofermenters, stimulated by the Factor L but never favored by the Factor T.

Table 3. Grouping of the Strains of Rod-Formed Hiochi-Bacteria.

	Heterofermenters	Homofermenters
Exacting toward Hiochic Acid, 'True Hiochi-bacilli'	H 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12–1, 19, 24, 27, 28, 29, 35–4, 36, 37, 41, 44, 45, 46, 47, 48, 49, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 63, 64, 65, 66, 70, 71, 72, 75 .....(44 strains)	H 12–3, 14, 17, 42, 40, 43, 74 ..... (7 strains) H. 13, 15, 16 ..... (3 strains) H. 38 ....(1 strains)
Independent of Hiochic Acid, 'Hiochi-Lactobacilli'	H. 34, 50, 60, 67 ..... (4 strains)	H. 7, 68, 76, 26 .. (4 strains)

H-38 also requires Factor L as an essential nutrient, but the stimulatory effect of Factor T for this strain is rather obscure. From the experimental data on this strain, a supposition may arise that some other growth factor or

factors might exist in saké. Eventually, homofermentative hiochi-bacteria can thus be divided into three groups, two of which are quite identical with the respective groups of heterofermenters, but in this case a transitional group, for which the Factor T acts more or less stimulatorily, should be inserted between the two groups.

Nutritional requirement of the spherical strain is quite similar to that of the transitional group of homofermenting rods.

Hiochi-bacteria, whatever they may be homo- or hetero-fermenters, require some factors originating from liver and/or peptone (Factor L), probably of peptidic nature. However, they can be classified as shown in Table 3, by the requirement toward hiochic acid, which exists in saké.

Groups which are not exacting for the hiochic acid are nothing but the so-called 'hiochi-lactobacilli', because they can grow in ordinary culture

Table 4. Effect of Starting pH.

		Representative strain	Number of analogous strains	ml. of *N*/10 NaOH per 2 ml. of media, whose starting pH were:					
				4.0	4.5	5.0	5.5	6.0	7.0
Hetero-fermenters	'True Hiochi-bacilli'	H 54	2	0.97	0.72	0.79	0.37	0.12	0.20
		H 1	5	0.32	2.40	2.39	1.12	0.70	0
		H 6	12	0.18	1.21	2.30	0.91	0.35	0
		H 3	10	0.23	1.37	2.38	2.16	0.14	0.18
		H 2	8	1.39	2.08	2.20	2.29	0	0
		H 28	2	0.14	1.99	2.21	1.82	2.03	0
	'Hiochi-lacto-bacilli'	H 50	3	0.59	2.02	2.21	2.09	1.98	1.01
		H 67	1	0.37	1.97	2.02	2.15	1.92	2.19
Homo-fermenters	'True Hiochi-bacilli'	H 42	2	0	1.13	1.41	0	0	0
		H 14	1	0.10	1.20	2.75	0.09	0	0
		H 12–3	1	0	0.84	1.51	2.18	0	0
		H 17	2	0	2.00	2.72	3.16	0	0
	Transitional Strains	H 16	1	0	2.15	2.12	0	0	0
		H 38	1	0	0.12	2.01	0.23	0	0
		H 13		0	2.73	3.93	2.82	0	0
	'Hiochi-lacto-bacilli'	H 68	3	0.24	0.17	1.54	3.42	3.41	3.95
Spherical strain		H 35–1	1	0	0	0	1.77	1.52	2.33
L. acidophilus				0.51	0.98	2.11	2.53	2.11	1.28
L. plantarum				0.48	1.35	2.13	2.24	2.84	3.49
L. brevis				0	0	0.48	1.00	1.71	1.83

Culture medium: 'Polypeptone' 5 g, yeast extract 5 g, liver infusion 200 ml., saké 200 ml., glucose 20 g, Na acetate 20 g, KH_2PO_4 10 g in 1 liter.

media such as glucose yeast water, or glucose bouillon. On the other hand, the groups which are exacting toward hiochic acid correspond to the 'true hiochi-bacilli'. Here we think, that the basis and the appropriateness of the old argument proposed by TAKAHASHI has been clarified.

Effect of pH upon the Reproduction of Hiochi-Bacteria: The most favorable pH value for reproduction of ordinary lactic acid bacteria lies about 6–7. But, since they produce lactic acid simultaneously with their growth in sugary media, their activities may be lost in due time. So, for keeping them always fully active, we are accustomed to use suitable neutralizing agents such as $CaCO_3$. Most of hiochi-bacteria, on the contrary, cannot grow in nutritive media containing $CaCO_3$, and for making them grow, the initial pH of the media must be controlled below 5.5.

For demostrating this, each one drop of bacterial culture was inoculated to 2 ml of media which had been adjusted to various pH values. After 7–10 days' incubation, degree of reproduction was compared by the acidity of the media by alkali titration (Table 4).

Though the favorable pH range are somewhat different in different strains,

Table 5. Effect of Temperature.

		Strain	ml. of $N/10$ NaOH per 2 ml.				
			25°C	30	37	40	45
Hetero-fermenters	'True Hiochi-bacilli'	H 1	2.00	2.20	0	0	0
		H 5	2.00	2.10	0.05	0	0
		H 44	2.02	2.04	0.10	0	0
		H 63	2.00	2.05	0.15	0.10	0
		H 27	2.04	2.05	2.05	0	0
	'Hiochi-lactobacilli'	H 34	1.90	2.00	1.90	1.70	0
		H 67	1.90	2.10	2.00	1.80	0
Homo-fermenters	'True Hiochi-bacilli'	H 14	3.21	3.70	2.50	2.60	0
		H 17	3.70	3.62	3.47	0.45	0
		H 40	3.52	3.71	2.66	2.09	0
		H 42	3.00	3.10	2.45	2.34	0
		H 43	2.23	2.69	2.13	2.07	0
	Transitional strains	H 15	2.86	2.80	0.80	0	0
		H 38	2.38	2.10	0.10	0	0
	'Hitochi-lactobacilli'	H 68	4.00	3.90	4.05	4.00	0.15
		H 26	2.60	2.60	2.40	1.90	0
Spherical strain		H 35–1	1.70	1.60	0	0	0

Culture medium: same as in Table 4, pH is adjusted to 5.5.

most of hiochi-bacteria are markedly acidophilic in contrast to ordinary lactic acid bacteria. 'Hiochi-lactobacilli', however, are quite alike with ordinary lactic acid bacteria in their favorable pH ranges, too.

Whichever they may be homo- or hetero-fermenters, 'true hiochi-bacilli' grow and reproduce most vigorously at pH 5.0, but as a rule they cannot grow at pH 6.0 or above. Whereas 'hiochi-lactobacilli' can carry on their vitalities in nearly neutral reaction. The pH range of the transitional strains is quite similar to that of 'true hiochi-bacilli'.

Effect of Temperature upon the Growth of Hiochi-Bacteria: Experimental results on the temperature-relationships of hiochi-bacteria are shown in Table 5.

Heterofermentative 'true hiochi-bacilli' are comparatively thermolabile. They can never grow at 40°C, and in addition some of these become inactive at 37°C.

Other groups are practically similar in their temperature relationships, viz. most strains can grow at 40°C, but never at 45°C.

In general, hiochi-bacteria may be concluded as mesophilic.

SUMMARY

1) Sixty-four strains of hiochi-bacteria isolated from spoiled saké have been used for this research.

2) Hiochi-bacteria have been recognized to belong to the mesophilic lactic acid bacteria. They can be divided into homofermenters and heterofermenters, and each of these two groups can be further classified by whether they demand hiochic acid or not.

3) This system of classification of hiochi-bacteria is apparently identical with the combination of the classical systems of Yamasaki and of Takahashi, who classified by plus or minus of gas-formation, and plus or minus of growth in the media other than saké, respectively.

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TAXONOMIC STUDIES ON THE HIOCHI-BACTERIA, SPECIFIC SAPROPHYTES OF SAKE
II. IDENTIFICATION AND CLASSIFICATION OF HIOCHI-BACTERIA

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In the previous paper[1] it was concluded that the hiochi-bacteria, both the so-called 'true hiochi-bacilli' and the 'hiochi-lactobacilli', belong to the category of lactic acid bacteria. The question arose then as to what systematical positions are occupied by these hiochi-bacteria. Important characteristics for determining the systematical position of lactic acid bacteria are the type of fermentation (such as homo or hetero), optical property of the lactic acid produced and the type of sugars which are fermented by the bacteria.

FERMENTABLE SUGARS AND OPTICAL PROPERTY OF LACTIC ACID.

Using culture medium IV (described in the preceding paper) in which glucose was substituted by various sugars, the fermentable sugars of 64 strains of hiochi-bacteria were examined. The results are summarized in Table 1, together with the optical properties of lactic acid which were investigated in our previous study.

Heterofermenters.

Among heterofermenters, three strains disignated as H-50, H-34 and H-60, which were hiochic acid-independent, showed fermentation activity with glucose, fructose, galactose, sucrose, xylose, and α-methyl glucoside, producing inactive lactic acid. Dextran formation in sucrose-gelatin could not be observed, but mannitol was formed distinctly when fructose was used as substrate. In view of these characteristics, these may be regarded as being identical with *Lactobacillus fermentum* Beijerinck α-type Kitahara[2], although the optimum temperature for these strains is a little lower than that for typical strains of *L. fermentum* α. Strain H-67 is somewhat different from the above mentioned three strains, in being incapable of fermenting the sugars other than monosaccharides, although it can ferment xylose. Considering the slightness of difference, this strain may be classed in the same species.

All 44 strains of heterofermentative 'true hiochi-bacilli' were found to be totally incapable of fermenting sugars tested in this experiment except glucose and fructose.

Table 1. Fermentable Sugars and Optical Forms of Lactic Acid.

	Heterofermenters				Homofermenters								Spherical strain
	'True hiochi-bacilli'		'Hiochi-lactobacilli'		'True hiochi-bacilli'		Trans-itional strains		'Hiochi-lacto-bacilli'				
Representative strains	H 5	H 46	H 67	H 50	H 14	H 40	H 13	H 38	H 7	H 76	H 26		H 35–1
Number of analogous strains	42	2	1	3	4	3	3	1	2	1	1		1
Xylose	−	−	+	+	−	−	−	−	−	−	−		+
Arabinose	−	−	−	−	−	−	−	−	−	−	+		+
Rhamnose	−	−	−	−	−	−	−	−	−	−	−		−
Glucose	+	+	+	+	+	+	+	+	+	+	+		+
Fructose	+	+	+	+	+	+	+	+	+	+	+		+
Mannose	−	−	−	−	+	+	+	+	+	+	+		+
Galactose	−	−	−	+	−	−	−	−	+	+	+		+
Maltose	−	−	−	+	+	+	+	+	+	+	+		+
Sucrose	−	−	−	+	−	−	−	+	+	+	+		+
Lactose	−	−	−	−	−	−	−	−	−	+	+		−
Raffinose	−	−	−	+	−	−	−	+	−	−	−		+
Dextrin	−	−	−	−	−	−	−	−	−	−	−		−
Dextran	−	−	−	−	−	−	−	−	−	−	−		−
Inulin	−	−	−	−	−	−	−	−	+	−	−		−
Starch	−	−	−	−	−	−	−	−	−	−	−		−
Glycogen	−	−	−	−	−	−	−	−	−	−	−		−
α−Methyl glucoside	−	−	−	+	−	−	−	−	(+)	+	−		+
Salicin	−	−	−	−	+	+	+	−	+	+	+		−
Glycerol	−	−	−	−	−	−	−	−	−	−	−		−
Optical form of lactic acid	DL	L	DL	DL	D	DL + D	DL + D	DL + L	DL + L	DL + L	DL		D

As they do not ferment sucrose, the dextran formation could not be examined. It is concluded that they belong to α-typed hetero-lactic acid bacteria, because they have the ability to change over 30 % of fructose to mannitol (Table 2). As a rule they produced DL-lactic acid. It is worth while to mention here that L-lactic acid was formed by two heterofermenting rods, H-45 and H-46. This phenomenon offers a serious problem pertaining to the classification of lactic acid bacteria, but we will reserve the discussion on this problem for some future occasion.

Table 2. Fermentation of Fructose by Heterofermentative Hiochi-Bacteria. Culture: 2.5 g fructose/300 ml., 30°C, 7 days.

Strain	Sugar consumed	Products				Remarks
		Mannitol		Lactic acid	Acetic acid	
	g	Quantity g	Yield %	g	g	
H 34	2.40	0.64	25.8	0.64	0.26	In the presence of $CaCO_3$
H 60	2.40	0.68	28.3	0.56	0.22	
H 1	2.34	0.76	32.4	0.46	0.31	In the absence of $CaCO_3$ Initial pH: 5.0
H 11	2.40	0.72	30.0	0.49	0.32	

There may be some reason to assume that these strains might be mutants of *L. fermentum* which had been simplified in their fermentative ability by adapting to limited nutritional environments[3]. This assumption seems, however, to be inadequate when we consider that these strains appear most frequently with vigorous growth activity in saké which can hardly be regarded as a poor nutritive medium. Thus the present authors want to propose a name *Lactobacillus heterohiochii* nov. sp. from the meaning of heterofermentative hiochi-bacteria.

Strains belonging to Type AF 1 in YAMASAKI's[4] collection are probably identical with this species. This species has appropriately been used for biological determination of hiochic acid[5].

Homofermenters.

Among homofermenters, strain H-26 ferments maltose, sucrose, lactose, arabinose, and mannitol besides hexoses and forms DL-lactic acid. So it may be identified as a typical strain of *Lactobacillus plantarum* (ORLA-JENSEN) HOLLAND.

Two strains, H-7 and H-68, have no ability to ferment mannitol. There may be some reason to consider these strains as *Lactobacillus japonicus* KITAHARA et ISHIDA[6], but it will be more probable to identify them as *Lactobacillus acidophilus* (MORO) HOLLAND which lost the fermentability of lactose, as they produce DL+L-lactic acid instead of DL- or DL+D-acid which characterizes *L. japonicus*[7]. This conclusion is reinforced by the fact that

there exists another strain, H–76, which is identified as a typical strain belonging to *L. acidpohilus*. This strain retains the abilities of lactose fermentation and of milk-coagulation, but in other properties it cannot be differentiated from above mentioned two strains. *Lactobacillus hyochi* OTANI[8] may probably be a synonym of this species. It is noteworthy that H–7 had a strong fermentability of inulin. Though this property is not useful for classifying lactic acid bacteria, except in the case of *Streptococcus inulinaceus*, we are occasionally surprised by the phenomenon on the way of examining hundreds of newly isolated strains.

Seven strains, which require hiochic acid as an essential nutrient, ferment glucose, fructose, mannose, maltose, salicin and mannitol, and produce laevo-rotatory lactic acid, i.e. pure or partly racemized D-lactic acid.

Species belong to so-called Thermobacterium (ORLA-JENSEN)[9], such as *L. lactis* and *L. delbrückii* (Syn. *Thermob. cereale*), which can grow and ferment actively at 48–50°, are well-known as D-lactic acid forming homofermenters. Hiochi-bacteria, however, can never be included in *Thermobacterium* from their growth temperature range. So these strains should be considered very curious. Only one species, *Lactobacillus leichmannii* HENNEBERG[10], has been known as D-forming mesophilic homofermenter.

We acquired *L. leichmannii* ATCC 4797, and ATCC 7380, as strains for determining vitamin B_{12}. Here, we used these strains and an authentical strain of *L. delbrückii* for comparing with the D-forming hiochi-bacilli. Table 3 shows the summary of the experiments.

Table 3. Comparison of the Characteristics of Homofermentative 'True Hiochi-Bacilli' with Those of *L. leichmannii* and *L. delbrückii*.

	Acid-formation from:											Optical form of lactic acid	Long and twisted cells	Optimum temp.	Maximum temp.	Optimum pH
	Glucose	Fructose	Mannose	Galactose	Maltose	Sucrose	Lactose	Pentoses	Salicin	Mannitol	Dextrin			°C	°C	
H 14 and analogous 6 strains	+	+	+	−	+	−	−	−	+	+	−	D	−	30	40	5.0
L. leichmannii ATCC 4797	+	+	+	+	+	+	+	−	−	−	−	D	+	37	48	6.5
L. leichmannii ATCC 7380	+	+	+	−	+	+	+	−	−	(+)	(+)	D	+	37	50	6.5
L. delbrückii	+	+	+	+	+	+	−	−	−	−	−	D	+	40	52	6.5

All strains coincide with one another in the character of forming D-lactic acid. This group of hiochi-bacteria cannot ferment sucrose which *L. leichmannii*

very readily can. On the other hand, the former can readily ferment mannitol and salicin, upon which the action of the latter is very limited or completely lacking. *L. leichmannii* is rather akin to *L. delbrückii*, that is Thermobacterium, not only in fermentable sugars but also in the temperature relationship and morphological nature.

In BERGEY's Manual, *L. leichmannii* is described as to be very similar to *L. delübrckii* except the lower optimum and maximum growth temperatures. It is a matter of regret that optical property of lactic acid is not recorded in the article of *L. leichmannii*.

We have met a typical strains of *L. leichmannii*, but at least from our results of comparative experiments, this species is more likely included in the Thermobacterium. Our hiochi-bacteria are, on the contrary, obviously mesophilic.

Furthermore, cells of *L. leichmannii* and *L. delbrückii* are, as described by HENNEBERG[11], occasionally markedly elongated and twisted, but in the hiochi-bacteria, such morphological changes could not be observed. It can be added as a difference that, because hiochi-bacteria of this type cannot grow in neutral media, fermentation cannot be carried out, unlike ordinary lactic acid bacteria, in the presence of $CaCO_3$.

The present authors want to propose a name *Lactobacillus homohiochii* nov. sp. to this type of hiochi-bacteria, from the meaning of homofermentative hiochi-bacteria. YAMASAKI's strains of the Type AF-100 may be included in this species. This species can also be utilized for determination of hiochic acid as well as *L. heterohiochii*.

Among the four transitional strains, three strains, H-13, H-15 and H-16, are very close to the above mentioned 'true hiochi-bacilli', except in a slight difference in nutritive requirements. So that these strains are preferably included in *L. homohiochii*.

Another strain H-38 is further different from *L. homohiochii* not only in the nutritive requirement, but also in fermentable sugars, that is, this strain can ferment sucrose and raffinose on one hand, but cannot ferment mannitol and salicin on the other hand. There is no species into which this strain is suitably admitted. The authors, however, prefer to think it as a variety of *L. homohiochii*. *L. filamentosus* and *L. alcoholiphilus* described by OTANI are also to be considered as transitional type of *L. homohiochii*.

Spherical strain.

One strain of spherical shape which was exceptionally isolated from spoiled sake, carried out heterofermentation and produces D-lactic acid.

It is then easily concluded, even without examining its morphological characters of forming beaded chains, to be a member of Leuconostoc.

This strain produces dextran from sucrose and readily ferments pentoses, so that it is identified as *Leuconostoc mesenteroides* (CLENKOWSKI) VAN

Table 4. Brief Classification of Lactic Acid Bacteria.

A. GRAM-positive.
B. Spheres.
C. Catalase negative.
D. Homofermenters.
E. Racemiase negative (L-formers). .....Genus **Streptococcus**
Sc. cremoris, Sc. thermophilus,
Sc. lactis, Sc. bovis, Sc. inulinaceus, Sc. faecalis,
Sc. glycerinaceus, Sc. liquefaciens.
EE. Racemiase positive (L+DL~DL-formers)..Genus **Pediococcus**
Pc. henrebergi, Pc. lindneri.
DD. Heterofermenters (D-formers) .....Genus **Leuconostoc**
Leuc. dextranicum, Leuc. mesenteroides α,
Leuc. mesenteroides β.
CC. Catalase positive (L-formers).....Genus **Tetracoccus**
Tc. liquefaciens.
BB. Rods.....Genus **Lactobacillus**
C. Catalase negative
D. Homofermenters .....Subgenus True-Lactobacillus
E. D-Formers.
F. Racemiase negative or slightly positive.
(D−~D+DL-formers).....*Delbrückii*-series
L. delbrückii, L. lactis, L. leichmannii,
L. homohiochii.
FF. Racemiase positive (DL-formers)..... *Plantarum*-series
L. japonicus, L. plantarum (Syn.
L. arabinosus)
EE. L-Formers.
F. Racemiase positive.
(DL−~DL+L-formers). .....*Acidophilus*-series
L. acidophilus, L. saké, L. casei.
FF. Racemiase negative (L-formers). .....*Bulgaricus*-series
L. bulgaricus, L. xylosus.
DD. Heterofermenters.....Subgenus Beta-Lactobacillus
E. Racemiase positive (DL-formers).....*Brevis*-series
L. brevis α, L. brevis β, L. fermentum α,
L. fermentum β, L. heterohiochii.
EE. Racemiase negative (D-formers).....*Batatas*-series
L. batatas L. fructosus[12].
CC. Catalase positive (L-formers).....Subgenus Wild-Lactobacillus
L. thermophilus, L. ciliatus,
Microbacterium ORLA-JENSEN, So-called
spore-forming lactic acid bacteria.
AA. GRAM-negative (D-formers). .....Genus **Escherichia**
E. acidi lactici.

Tieghem.*

Positions of Hiochi Bacteria in the Classification of Lactic Acid Bacteria.

The location of the hiochi-bacteria in the classification of lactic acid bacteria may be made clear, if they are inserted in the newly proposed system of lactic acid bacteria (Table 4) by one of the present authors (Kitahara)[13].

DESCRIPTION OF NEW SPECIES

Lactobacillus heterohiochii nov. sp.

Round-ended rods, ordinarily 0.7–0.8 μ by 2–6 μ, but occasionally longer cells having 10–20 μ can be seen. Occurring singly, in pairs or in chains. Always Gram-positive, non spore-forming, and non motile. (Fig. 1)

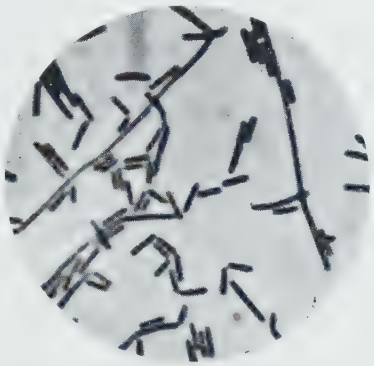

Fig. 1.
L. heterohiochii
×1,350
Liver extract-saké medium
30°C, 12 days.

Solid culture: Colonies in agar media are white glistering pinhead or fist-form. Cells composing the colony are strongly congealed with each other. Stab culture: Filiform or papillate growth, uniformly along the stab-canal, can be seen after 3–5 days, but no surface growth. Streak culture: As a rule no visible growth, but exceptionally slight growth can be observed.

Liquid culture: Liquid medium becomes somewhat turbid after 2–4 days, and reveals silky luster when being shaken. Afterwards a grayish white sediment is gradually formed. When old, the turbidity fades away and there remains a firm sediment at the bottom of the clear liquid.

It does not grow in ordinary culture media, such as glucose-yeast water, glucose bouillon, or milk, but does grow readily in saké. This is due to the essentiality of hiochic acid for the growth of the species. The hiochic acid is considered as a specific constituent of saké.

* A type of microorganism belonging to this species has been known as one of the important acidifiers in the cultivation of '*moto*', yeast starter for saké brewing. But owing to the differences in alcohol-resistance and arabinose fermentation, there may be no use to have such a fear that the useful organism for preparation of saké occasionally take a bad turn and attack the newly produced beverage.

Microaerophilic, catalase negative, nitrate not reduced to nitrite, and indole not formed. Readily ferments glucose and fructose, but cannot ferment other carbohydrates such as mannose, galactose, pentoses, oligosaccharides, polysaccharides, sugar alcohohols, and glucosides.

Glucose fermentation: Considerable amounts of ethanol, acetic acid and CO_2 are formed besides lactic acid. The yield of lactic acid is about 50 % of sugar fermented. The lactic acid is generally of pure DL-form.

Fructose fermentation: Generally, mannitol is produced as a chief product. Lactic acid, acetic acid, CO_2 and more or less ethanol are also produced.

Growth temperature: Optimum temperature is about 30°C, maximum temperature for the growth is considered to be lower than 40°C.

pH for growth: Markedly acidophilic, optimum pH being 5.0; can grow slightly at pH 6.0, and can never grow at pH 7.0.

Strongly resistant against ethyl alcohol, viz. 13–16 vol. % or more. Willingly grows in saké and renders it undrinkable. This phenomenon is called 'hiochi'.

An overwhelming species which appears as hiochi-bacteria.

Habitat: Spoiled saké.

Lactobacillus homohiochii nov. sp.

Round-ended rods, with 0.7–0.8 μ in breadth and as a rule 2–4 μ and occasionally 6 μ in length, the cells longer than 6 μ can hardly be observed (Fig. 2).

Fig. 2.
L. homohiochii
×1,350
Liver extract-saké medium
30°C, 12 days.

GRAM-positive, non-spore-forming, and non-motile.

Characters in solid and liquid media, as well as the nutritive requirement toward hiochic acid, are almost identical with those of the foregoing species.

Microaerophilic, catalase negative, nitrate not reduced to nitrite, indole not formed. Ferment glucose, fructose, mannose, maltose, salicin and mannitol, whereas galactose, sucrose, lactose, raffinose, pentoses, polysacchrides, α-methyl glucoside, and glycerol are as a rule not fermented.

Almost quantitative amount of lactic acid is formed from hexoses. The

optical form of the lactic acid is more or less laevorotatory, viz. D- or D+DL-lactic acid.

Growth temperature: Mesophilic, grow and ferment most vigorously at 30°C, generally somewhat at 40°C, but never at 45°C.

pH for growth: Acidophilic, optimum pH lies about 5.0, slightly active at pH 5.5, no growth at 6.0 or higher pH values.

Strongly resistant against ethyl alcohol, viz. 13–16 vol. %. Willingly grows in saké and makes it to 'hiochi'.

One of the chief species of hiochi-bacteria.

Habitat: Spoiled saké.

SUMMARY

Further examinations have been carried out for identification and classification of hiochi-bacteria, from the stand point of lactic acid-bacteriology.

The characteristics of 'true hiochi-baclli', both homo- and hetero-fermenters, do not coincide with those of any species which have hitherto been known. So the authors have proposed the names *Lactobacillus homohiochii* and *L. heterohiochii* nov. sp. respectively.

The 64 strains used throughout this research are classified as follows:

Spheres: Heterofermenting D-lactic acid former.
....*Leuconostoc mesenteroides* (1 strain).

Rods: Heterofermenting, as a rule DL-formers.
Hiochic acid independent, xylose is fermented.
....*Lactobacillus fermentum* (4 strains).
Hiochic acid essential, ferment only glucose and fructose.
....*Lactobacillus heterohiochii* n. sp. (44 strains)

Homofermenting
Hiochic acid independent, pentose-fermenting, DL-former
....*Lactobacillus plantarum* (1 strain).
pentoses not fermented, DL+L-former
....*Lactobacillus acidophilus* (3 strains).
Hiochic acid promotive, D+DL-formers.
....*Lactobacillus homohiochii* varieties (4 strains).
Hiochic acid essential, D- or D+DL-formers
....*Lactobacillus homohiochii* n. sp. (7 strains).

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EFFECT OF PHOSPHORESCENT PARTICLES SUSPENDED IN THE MEDIUM UPON THE EFFICIENCY OF LIGHT-ENERGY UTILIZATION BY *CHLORELLA* CELLS

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As is well known the efficiency of light-energy utilization by green cells in their photosynthetic and growth processes is a function of light intensity, and indeed it decreases rapidly with the increase of light intensity. The efficiency can, therefore, be increased if the light supplied to the cells is in some way, either spatially or temporally, "diluted". Kok (1) and Phillips et al. (2) have demonstrated that the efficiency of light-energy conversion in the growth of *Chlorella* was markedly increased when the culture was intermittently illuminated with flashing lights of adequate frequency and intensity. This result may be interpreted as being due to the situation that the intermittency of light had the effect of "diluting" strong flashing lights to weaker lights of longer duration (see Tamiya (3)).

Dealing with such an object as a suspension of *Chlorella* cells, we may expect that the "dilution" of light, and therefore the increase of the rates of photosynthesis and growth would also be achieved by suspending particles of an adequate phosphorescent substance in the medium. The effect, if any, may be seen most clearly when the population density of algal cells in the medium is sufficiently high. Described herein are the results of such experiments using *Chlorella ellipsoidea* as the experimental organism.

EXPERIMENTAL

Phosphorescent substance employed. The phosphorescent substance used was "copper-activated Zn-Cd-S-phosphor".* The powder of the substance is yellowish white in color, and when illuminated with visible light (violet to blue-green) emits light at 560-570 mμ with a half-life of 2–3 minutes and with a quantum efficiency of about 70 percent. Average size of the particles

* The writer takes this opportunity warmly to thank Professor Shoji Makishima of the Institute of Science and Technology, University of Tokyo, for supplying us with a sample of the substance and many valuable informations pertaining to it.

was 1.5 to 5 microns in diameter, a dimension that is comparable to that of algal cells. The quantity of the particles added to the algal suspension was 2.5 to 20 grams per liter.

Effect upon the rate of growth. Effect of the phosphorescent particles upon the rate of "linear growth"** of *Chlorella* was investigated with the following arrangement. Algal cells were suspended, with or without addition of the phosphorescent substance, in a medium containing, per liter: 3.0 gm. urea, 1.25 gm. KH_2PO_4, 2.5 gm. $MgSO_4 \cdot 7H_2O$, 0.003 gm. $FeSO_4 \cdot 7H_2O$, 0.037 gm. ethylenediamine tetraacetic acid (di-sodium salt), 1 ml. Arnon's "A5" solution (4). Five hundreds ml. each of the cell suspension were placed in an oblong flat flask described in our previous papers (5,6), (600 ml. in capacity with the inner thickness of 2.8 cm.), and under constant aeration with CO_2-enriched air, the culture was illuminated with light of 25 or 50 kilolux (incandescent lamps). The rate of introducing CO_2-enriched air was 200 to 300 ml. per minute, which was sufficient to keep both algal cells and phosphorescent particles suspended homogeneously in the medium. The initial concentration of algae was 10 ml. (packed volume) per liter, a concentration which was high enough to cause the total absorption of incident light. The culture lasted 5 days at 25°, and the rate of growth was determined by measuring the daily increase of the packed cell volume per liter of culture. The results obtained are shown in Table I, from which it appears that the presence of the phosphorescent particles in the medium had no effect upon the rate of algal growth.

Table I. Effect upon the Rate of "Linear" Growth.

Concentration of phosphorescent substance added	Average rate of growth (in ml./lit./day) occurring under the light of	
	25 kilolux	50 kilolux
0	6.75	8.90
0.25%	6.65	8.80
0.50%	6.85	8.90

Effect upon the rate of photosynthesis. Manometric experiments were run to examine the effect of the substance upon the rate of photosynthesis. Algal cells ("dark cells"***) were suspended in a 15-ml. aliquot of Warburg's carbonate mixture No. 9, in the concentration of 10 to 32 ml. (packed volume) per liter. The suspension was vigorously agitated by rotating the vessel at 250-300 r.p.m. in a circle of 10 mm. in diameter. The thickness of algal suspension in the vessel was about 1 cm. The rate of photosynthetic O_2-output was determined by taking into consideration the rate of respiration in the dark.

** The growth occurring when the population density is sufficiently high (5).

*** See our previous papers (6,7).

The experiment was repeated several times, but in no case was appreciable effect of phosphorescent particles detected. The data for a typical experiment are presented in Table II.

Table II. Effect upon the Rate of Photosynthesis.
Concentration of algae: 32 ml. per liter; Temperature: 25°.

Concentration of phosphorescent substance added	Rate of photosynthesis (in ml.O_2/hr./gm. of cells) occurring under the light of	
	13 kilolux	30 kilolux
0	7.9	15.1
0.25%	8.1	15.0
0.5%	7.5	16.0
1.0%	7.4	14.5
2.0%	7.9	14.8

DISCUSSION

For both growth and photosynthetic rates, there was no sign of acceleration caused by the addition of phosphorescent particles in the medium. For the interpretation of the negative results obtained, the following two possibilities may be considered:

(i) The favorable effect of phosphorescence might have been canceled by the effect of shading of algal cells caused by the particles.

(ii) Owing to the high population density of algal cells, the average time of exposure of the particles to the light might have been too short to bring about full excitation of the phosphorescent substance.

SUMMARY

Using *Chlorella ellipsoidea* as material, investigations were made to examine whether the rates of growth and photosynthesis of algal cells (in high population densities) could be accelerated by the effect of phosphorescent particles ("copper-activated Zn-Cd-S-phosphor") suspended in the medium. It was revealed that, at least with the experimental arrangement adopted, there was neither accelerating nor suppressing effect attributable to the presence of the substance.

AKNOWLEDGEMENT

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STUDIES ON THE CONIDIAL COLOR CHANGE IN ASPERGILLUS FUNGI

PART I. PHYSIOLOGICAL MODIFICATION OF A GENETIC BLOCK BETWEEN YELLOW AND GREEN COLOR DEVELOPMENT

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It has been demonstrated by Iguchi[1], Ishitani and Sakaguchi[2] that the green strains of *Aspergillus sojae* and *Aspergillus oryzae* produced yellow and white offsprings when the conidia were treated with mutagenic agents such as ultraviolet light, X-ray or nitrogen mustard. The evidences that this change is a real genetic one have also been discussed in the previous paper.[3]

This paper deals with the studies which were carried out with an attempt to elucidate the physiological or biochemical meaning of the gentic block which might have occured in a step between yellow and green color development. The environmental conditions essential for the color change form yellow to green were pursued using a yellow mutant of *Aspergillus sojae* as the test organism. The results of studies on the chemical nature of the yellow and the green pigments will be published later.

EXPERIMENTAL

1. *Strains*

Yellow mutant Y_1[1b]: This mutant was derived from *Asp. sojae* 260*, a green strain, by irradiating the conidia with ultraviolet light. Strictly speaking, the color of conidial head is Yellow Ocher. The heterocaryon produced by the mixed culture of this strain and the white mutant of the same origin segregated into yellow, white and green. Therefore, the strain Y_1 was apparent to be a genuine mutant in genetic sense.

The white mutant $A1_4$ was also derived from *Asp. sojae* 260 with ultraviolet irradiation. The orign and the history of the other strains are recorded in Table 1 and Table 8.

2. *Media*

Koji extract medium: So-called solid Koji was prepared in ordinary way

* This strain is the same with *Asp. sojae* K.S.[3].

with the use of the steamed rice and *Asp. oryzae*. The extract contains about 8 per cent of sugars, mainly consisting of maltose, isomaltose and glucose. In this experiment, conidia of 7 days old culture on Koji extract agar slants were used.

Malt extract medium: Malt extract prepared in ordinary way was diluted to Ballg. 2°.

Synthetic medium: The basal composition of the medium was as follows: glucose, 1.8%; KH_2PO_4, 0.05%; $MgSO_4 \cdot 7H_2O$, 0.025% and nitrogen source, 0.05%. The initial pH was 5.4. With regard to the modified media, see text.

3. *Color change induced by culture filtrate and mycelial mat extract.*

As previously reported, the conidial color of some yellow mutants of *Asp. oryzae* and *Asp. sojae* does change from yellow to green in the presence of culture filtrate which was prepared from different strains[3]. The effects of culture filtrate and mycelial mat extract upon the color change were discussed again in this paper.

The filtrate and the mycelial mat extract were prepared in the following way.

Table 1. Effect of Culture Filtrates and Mycelial Mat Extracts upon the Conidial Color Change of Y_1 Strain.

Strains used for the preparation of filtrate and mycelial mat extract	Degree of Greenization*		
	Culture filtrate		Mat extract
	Without heating	With heating**	
Control (malt extract)	−	−	
Asp. sojae 260	++	++	+
Asp. sojae 260 mutant Al_4	++	++	++
Asp. niger BL-5-1	++	++	+
Asp. usamii R 0635	++	++	+
Asp. ochraceus 24-6	++	++	
P. frequntaus 1256	++～+++	++～+++	+

* With regard to these symbols, see text.

** Heating condition: 60 mins. at 100°.

The filtrate: Six different strains shown in Table 1 were inoculated separately, one loopful in each, into 200 ml. of the malt extract liquid medium in 500 ml. Erlenmyer flasks. The inoculated flasks were incubated for 5 days at 30°. The growth was filtered through a Seitz filter after the pH of the media was adjusted to 5.4 with 1N NaOH.

The mycelial mat extract: The mycelial mats, which were harvested from the cultures mentioned above and were washed three times with distilled water, were ground in a mortar with quartz sands. One hundred ml.

of distilled water were added to each of the ground mats. The mashes resulted were then heated for 60 mins. at 100°. The filtrate was called "mycelial mat extract."

The observation of color change was done as follows; a mixture of 10 ml. of the melted malt extract ager (Ballg. 2°) and an equal amount of the culture filtrate or the mat extract were solidified in separated dishes, and the yellow conidia of Y_1 strain were inoculated on them. After 5 days incubation at 30°, the color of the newly formed conidia were observed under the microscope at the magnification of 100×.

The rate of the occurrence of green conidial head was estimated by the following formula:

$$\% \text{ green conidial head} = \frac{\text{number of green conidial head} \times 100}{\text{total number of head counted}}$$

% green conidial head	corresponding symbols
0	−
<2	+
2~ 5	++
6~10	+++
>10	++++

At least 800 conidial heads were counted in each determination.

The results thus obtained are illustrated in Table 1. As seen in the Table, all of the culture filtrates and the mat extracts were proved to be effective in changing the color of conidia, although the degree of greenization was not so sufficient as to make it possible to draw a decisive conclusion. The effect of culture filtrates was not destroyed by heating the filtrate for 60 min. at 100°, indicating that the effective factor(s) is not heat-sensitive. It is noteworthy, that the culture filtarte or the mat extract of the white mutant Al_4 arising from the same origin was effective, while the culture filtrate coming from the Y_1 strain itself was ineffective.

With the intension to make the effect clearer, the authors have tried several times to isolate the effective factor(s) from the culture filtrate or the mat extract. However, the culture filtrate concentrated to 1/5 of the original volume did not show any increase in the degree of greenization.

4. *Color change induced by halogen and copper ions.*

In the above experiments, the degree of greenization caused by the addition of culture filtrate, mat extract or the concentrate of culture filtrate was usually less than 10 per cent. Therefore, it seemed hard to pursue the effective factor(s) in the culture filtrate or mat extract.

By several workers, it has been already reported that the conidial color of fungus is affected by the pH of medium and by the kinds and contents of heavy metals in the medium. For example, Bortels[4], Roberg[5],

Steinberg[6], Yosimura[7], Miwa[8] and Gollmick[9] have confirmed that the presence of copper ions in the medium is needed for the production of the characteristic color of the mold conidia in the cultures of *Asp. niger* or *Asp. oryzae*.

During the course of studies on the effects of the chemical composition of a synthetic medium on the conidial color of our tested organisms, it was found that both the copper and halogen ions are indispensable for the greenization of conidia in our strain Y_1. The experiments and the results are described below.

(1) *Color change in the presence of chlorine ions.*

Ten kinds of synthetic media were prepared to supply different nitrogen sources (Table 2). Approximately 2×10^4 conidia of Y_1 strain were inoculated into each of the test tubes containing 5 ml. of the media. After 5 days incubation at 30°, the degree of conidial color change was determined with the naked eye. In this and the subsequent experiments, a new grading system as described was adopted to represent the degrees of greenization.

% green conidial head	Symbols
0	−
1～ 20	+
21～ 40	++
41～ 60	+++
61～ 80	++++
81～100	+++++

Table 2. The Conidial Color Change of Y_1 strain in Synthetic Media Containing Different Nitrogen Sources.

Kinds of N-source	Growth*	Degree of Greenization**	General Appearance***
KNO_3	##	−	aniline yellow
$NaNO_3$	##	−	aniline yellow
NH_4NO_3	##	−	light cadmium
NH_4Cl	##	+++	javel green
$(NH_4)_2SO_4$	##	−	empire yellow
$(NH_4)_2CO_3$	##	−	light cadmium
$(NH_4)_2HPO_4$	##	−	aniline yellow
Ammonium tartarate	##	−	light cadmium
Urea	#	−	aniline yellow
Asparagine	#	−	empire yellow

* ##: normal growth.

** Hereafter, a new grading system for greenization was employed, see text.

*** RIDGWAY, R.: Color Standards and Color Nomenclature (1912).

Table 2 shows the general appearance of conidial head resulted from the different nitrogen sources. As seen in the Table, only NH_4Cl was effective in producing green conidia, whereas the other inorganic and organic nitrogen sources such as KNO_3, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, $(NH_4)_2CO_3$, $(NH_4)_2HPO_4$, ammonium tartarate, urea and asparagine were ineffective.

Since NH_4Cl was the only compound containing Cl ions among the nitrogen sources tested, it was thought that Cl ions from NH_4Cl might be effective. Therefore, the three kinds of media, in which the nitrogen was supplied with $(NH_4)_2HPO_4$ and the Cl ions were afforded in the form of either NaCl, KCl or $CaCl_2$, were then tested. The concentration of the chlorides was adjusted to 1/20 M.

Table 3. Effect of Chlorides upon the Conidial Color Change of Y_1 Strain.

Kinds of chloride*	Growth	Degree of greenization
no-addition	##	−
NaCl	##	++++
KCl	##	++++
$CaCl_2$	##	++++

* Final concentration is 1/20 M.

As shown in Table 3, all of the three chlorides were found to be effective in inducing greenization, although the effects were not so complete as those which appear in the later section of this paper.

Table 4. Effect of Halogen Compounds upon the Conidial Color Change of Y_1 Strain.

Final concentration M.	Degree of greenization		
	KCl	KBr	KI
1/2	−*	±*	−*
1/5	++++	+++++	−*
1/10	+++++	+++++	+++++
1/20	++++	++++	+++++
1/50	+	++	++++
1/100	−	±	++
1/200	−	−	−
1/500	−	−	−
1/1000	−	−	−

* Growth and sporulation were poor.

As the chlorides showed a marked effect, the effects of the other halogen compounds such as KBr and KI were also examined. The results were shown in Table 4. It is clear that both bromide and iodide are also effective in inducing greenization. From the comparison of the lowest concentrations required for greenization, it can be said that iodide was the most effective among the halogens tested.

(2) *Color change in the presence of copper and halogen ions.*

At this step of study, the authors thought of the importance of heavy metals in color development. Therefore, the following experiments were carried out.

As the synthetic medium so far used was found to contain about 10^{-6}M. of Cu, the synthetic medium containing $(NH_4)_2HPO_4$ as nitrogen source was treated with Ca-phosphate $(Ca_3(PO_4)_2)$ following the method described by Sakamura[10]. The content of Cu in treated medium was confirmed to be less than 10^{-8} M. when determined by diethlydithiocarbamate method[11]. The contents of other metals were not examined, but they were supposed to be the same order as that of Cu. To the metal deficient medium thus prepared, the sulfates of Cu^{++}, Fe^{++}, Mn^{++}, Zn^{++}, Ni^{++}, Co^{++} and Cr^{++} were added with a concentration of 10^{-5} M. The media lacking one of the above seven heavy metals were also prepared. In adition, these media were further subdivided into the two groups with and without the added KCl (0.1 M.). The initial pH of these media were adjusted to 5.4.

The strain Y_1 showed a normal growth in all of these media except the control to which no metals were added. The conidial color changes resulted are demonstrated in Table 5. From the results, it would be apparent that

Table 5. Effect of Heavy Metals upon the Conidial Color Change of Y_1 Strain.

Kinds of metals added into medium	Degree of greenization	
	Without KCl	With KCl
Control (metal deficient)	–	–
Complete*	–	+++++
Complete minus Zn^{++}	–	++++
〃 〃 Ni^{++}	–	++++
〃 〃 Cu^{++}	–	–
〃 〃 Fe^{++}	–	++++
〃 〃 Mn^{++}	–	++++
〃 〃 Cr^{++}	–	+++++
〃 〃 Co^{++}	–	++++

* Complete medium contains 10^{-5} M. each of Zn^{++}, Ni^{++}, Cu^{++}, Fe^{++}, Mn^{++}, Cr^{++} and Co^{++} sulphates.

in addition to the presence of Cl ions, the presence of added copper is needed for the green color development in the case of strain Y_1.

The optimum concentration of Cu^{++} for green conidia formation was then investigated. With the addition of Zn^{++}, Fe^{++} and Mn^{++} sulfates to aid full growth, and KCl to help greenization, the concentration of $CuSO_4$ in the media was graded into seven, i.e. 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M. Table 6 indicated that the optimum concentration of Cu^{++} for the conidial color change is 10^{-4} to 10^{-5} M. in the presence of 0.1 M. KCl.

Table 6. Optimum Concentration of Copper ions for the Conidial Color Change.

Concentration of $CuSO_4$ M.	Yellow mutant Y_1**		Original green strain** (*Asp. sojae* 260)	
	Growth*	Degree of greenization	Growth*	Degree of greenization
10^{-3}	##	−	##	+
10^{-7}	##	+	##	+++
10^{-6}	##	+++	##	++++
10^{-5}	##	++++	##	+++++
10^{-4}	##	+++++	##	+++++
10^{-3}	#	+++++	#	+++++
10^{-2}	±	±	±	±

* Symbols ## shows normal growth.

** The basal medium for yellow mutant Y_1 contains 0.1 M. KCl, but the medium for original strain contains no KCl.

In parallel with the study with Y_1 strain, the orignal green strain, *Asp. sojae* 260, was cultivated separately. The results obtained with this strain are shown in the right half of the Table 6. It is interesting to note that the green strain remains yellow in the absence of Cu ion, but turns into green

Table 7. Effects of the Concentrations of Cu^{++} and Cl^- ions upon the Conidial Color Change of Y_1 Strain.

Final concentration of KCl M.	Final concentration of $CuSO_4$ M.					
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
1/10	+++++*	+++++	+++++	+++	−	−
1/20	+++++	++++	++++	+	−	−
1/50	+++	+++	+	+	−	−
1/100	+	+	±	±	−	−
1/200	++	+	−	−	−	−

* Degree of greenization.

in the presence of Cu, without requiring the addition of Cl ions. Next, the effects of the various combination of Cu and Cl ions were then examined. As shown in Table 7, at the combinations of 1/10 M. of KCl and each 10^{-3}, 10^{-4} and 10^{-5} M. of $CuSO_4$ or at the combination of 1/20 M. of KCl and 10^{-3} M. of $CuSO_4$, the conidial color almost completely changed into green.

(3) *Other conditions for the formation of the green conidia.*

Besides the effect of Cu and Cl ions, the effects of some other conditions which affect the conidial color change were studied in the presence of two ions. The results are summarized in short:

Optimum pH: Using Mc Ilvaine's buffer (1/5 M. Na_2HPO_4 and 1/10 M. citric acid), the optimum pH for the color change was found to lie between 2.4 and 4.0.

Nitrogen source: In general, it was found that the nitrogen sources such as $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$ which would give acid reaction supporting the higher degree of greenization.

C/N ratio: Using glucose and $(NH_4)_2HPO_4$ as carbon source and nitrogen source, the optimum concentrations of glucose and ammonium salt were found to be 1/6 – 1/10 M. and 1/200 – 1/400 M., respectively. It is interesting that this C/N ratio was completely in accordance with that found by Ishitani and Sakaguchi[2,12] to be the most suitable for the anastomosis in fungi.

5. *Studies with other strains.*

In order to make the site of Cu and Cl actions clearer, the strains other than *Asp. sojae* 260 and its mutants were tested using Cu and Cl supplemented medium. As shown in Table 8, among the 26 kinds tested, only the yellow mutants of *Asp. sojae* and *Asp. oryzae* changed the conidial color, while the other color mutants such as white and brown of the same origins did not. This would indicate that both of Cu and Cl ions are specially related to the genetic block between yellow and green color development.

6. *Stability of green conidia thus obtained.*

Genetically speaking, the yellow mutants must return to yellow when they are transplanted to a ordinary medium such as Czapek or malt extract media even if some modifications might be made on different media. In order to confirm this possibility, the green conidia obtained by means of culture filtrate or Cu and Cl ions were plated on Czapek agar medium. It was found that all of the colonies appearing on the agar media were yellow just like those of Y_1 strain. This fact indicates that the change of conidial color from yellow to green was not a mutational one, but a tentative phenomenon. The term "physiological modification" was, therefore, given to this phenomenon.

Table 8. The Conidial Color Change by the Addition of Copper and Halogen Ions to the Medium in Several Other Strains of *Aspergillus*.

No.	Strains	Color of conidial head: Without the addition of Cu and Cl ions	Color of conidial head: With the addition of Cu and Cl ions
1	*A. sojae* 260 yellow mutant Y_1*	apricot yellow	javel green
2	〃 〃 〃 yellow mutant Y_2*	apricot yellow	javel green
3–4	〃 〃 〃 brown mutant B_1, B_2*	sudan brown	no change
5–11	〃 〃 〃 albino mutant Al_1～Al_7*	white	〃
12	〃 〃 〃 biochemical mutant H–1**	buckthorn brown	〃
13	〃 〃 〃 〃 〃 L–120**	yellow ocher	〃
14	〃 *oryzae* K. B. albino variant 122	white	〃
15	〃 〃 〃 yellow mutant K–13***	empire yellow	oil green
16	〃 〃 〃 〃 〃 K–8***	empire yellow	oil green
17	〃 *wentii* 23–2	orange citrine	no change
18–19	〃 *tamarii* T 16, 23–12	dresden brown	〃
20–21	〃 *ochraceus* 24–4, 24–6	warm buff	〃
22–23	〃 *candidus* PO, Bf	white	〃
24	〃 *terreus* 24–13	cinnamon	〃
25	〃 *flavipes* 26–5	avellaneous	〃
26	〃 *giganteus* albino mutant G–16****	white	〃

* Y_1, B_1, Al_1, Al_2, Al_3 and Al_4 strains were produced by UV treatment[1b]. Y_2, B_2 and Al_5 strains were produced by X-ray treatment[1c]. Al_6 and Al_7 strains were produced by nitrogen mustard treatment[1d].

** H–1 strain requires histidine and L–120 strains requires leucine, see *J. Agr. Chem. Soc. Japan* **29**, 682, (1955).

*** K–13 and K–18 strains were produced by UV treatment, see *J. Gen. Appl. Microbiol.* **1**, 272, (1955).

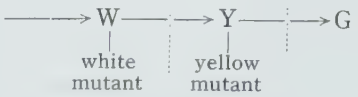
**** G–16 strain was produced by UV treatment, see *J. Gen. Appl. Microbiol.* **1**, 164, (1955).

DISCUSSION

It has been found that the yellow mutants of *Asp. sojae* and *Asp. oryzae* change their conidial color tentatively into green in the presence of culture filtrate or mycelial mat extract, or in the presence of added copper and halogen ions. This phenomenon of color change seems to occur commonly in the field of microbiology. However, the authors would like to point out the following specificities:

1. The fact that the original green strain remains yellow unless copper ions be present in the medium, and the yellow mutants require both, copper

and halogen ions for the color change suggested that copper ions are related to the intermediate step between yellow and green color development. According to the mutational and genetic studies so far published[3], the conidial color in *Asp. oryzae-sojae* group seems to proceed as follows:



Therefore, the tester strain Y_1 could be assumed to be a mutant in which the reaction between Y and G has been genetically blocked. In addition, it may be assumed that the co-existance of halogen ions with copper ions can restore above block. In yellow mutants, the ability of copper ion uptake must be reduced with the mutagenic treatments. It has been already known that copper ion is related to green conidia formation and the green strains contain a relatively larger amount of copper ions. For instance, Yoshimura[7] and Miwa[8] have reported the necessity of copper compounds for green conidia formation of *Asp. oryzae*, and Kikkawa and Ogita[13], and Searashi[14] pointed out that the green organisms or strains contained copper more aboundantly than yellow and white ones. Our date substantiated their finding. However, since the new fact that halogen ions (Cl, Br or I) are related to the green color development of yellow mutants was found in this paper, it can be assumed that halogen ions are directly correlated with copper ion uptake. We are now investigating the relationship between the halogen ion concentration in the media and the copper contents in green mycelial mat or green conidia.

2. In order to examine the postulated color change processes as described above, a simple experiment was made. When the yellow mutant Y_1 and the parent strain were inoculated into copper-deficient media with and without added halogen, respectively, the conidial color of both strains after 5 days incubation remained yellow. However, when copper ions were added to this 5 days culture, their conidial color did change from yellow to green during the subsequent incubation of 2 days.

On the other hand, it has been observed that the conidial color change during the growth of the parent strain proceeded form white to green via yellow, though the yellow stage was quite short. From these facts, it seems reasonable to suppose that the precursor of the green pigment in the conidia would be the yellow pigment.

3. With regard to the effect of culture filtrate or mat extract upon color change, it may be pointed out that the yellow mutant changed its conidial color with the addition of culture filtrate arising from the white mutant of the same origin. This fact is very suggestive when we compare this phenomenon with the green color formation in a heterocaryon in which both types of the cytoplasm co-exist in the same cell.

SUMMARY

1. Using genetically blocked yellow mutant of *Aspergillus sojae* 260, some environmental conditions which favor the conidial color change from yellow to green were investigated.

2. In the presence of culture filtrate or mycelial mat extract of other fungi, the yellow conidia of the Y_1 strain changed their color into green. However, the degree of greenization with these substances was not so sufficient as to make it possible to draw a decisive conclusion.

3. With the presence of copper and chlorine ions, the yellow color changed almost completely to green. The other halogens such as bromine and iodine could replace the chlorine ion. The color change was proved to occur only in yellow mutants of *Asp. sojae* and *Asp. oryzae*. Other mutants such as white and brown ones did not show such a phenomenon.

4. The green conidia thus obtained returned to yellow when they were transplanted on the ordinary media such as Czapek's agar and malt extract agar. It is apparent, therefore, that the color change was merely physiological modification.

5. For the development of green color, the original green strain required copper ion, but the yellow mutant required both copper and halogen ions.

6. The possible role of halogen ions and that of copper ions in inducing green conidia formation were discussed.

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BIOSYNTHESIS OF ^{14}C-LABELLED ADENINE AND GUANINE BY CHLORELLA ELLIPSOIDAE

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In the preceding paper (1), the author reported on the separation of uniformly ^{14}C-labelled amino acids from *Chlorella ellipsoidae* cultivated with ^{14}C-labelled carbon dioxide as the sole carbon source. The present paper describes the preparation of ^{14}C-adenine and guanine from the same source. The nucleic acids fraction (the radioactivity of which an equivalent to 15 % of Chlorella cell) was hydrolysed with acid and then the resulted bases were separated columnchromatography by ion exchange resin. The acid hydrolyzates were adsorbed on Dowex-1 (used as formate form) and eluted by formic acid, followed by formic acid-ammonium formate solution of various concentrations (2). The vases in the initial eluate were further run in Dowex-50 (used as chloride form) and eluted with hydrochloric acid (3). The results of two experiments were as follows. From 63×10^5 c.p.m. (2.2 %) of guanine were obtained in one experiment. In the other experiment, 23.6×10^5 c.p.m. (14 %) of adenine and guanine was obtained from 170×10^5 c.p.m.

PROCEDURE

1. Materials

The nucleic acid fraction was obtained from chlorella cell suspension after a modification of the method of W. C. Schneider (Table 1).

2. Acid hydrolysis of nucleic acid fraction

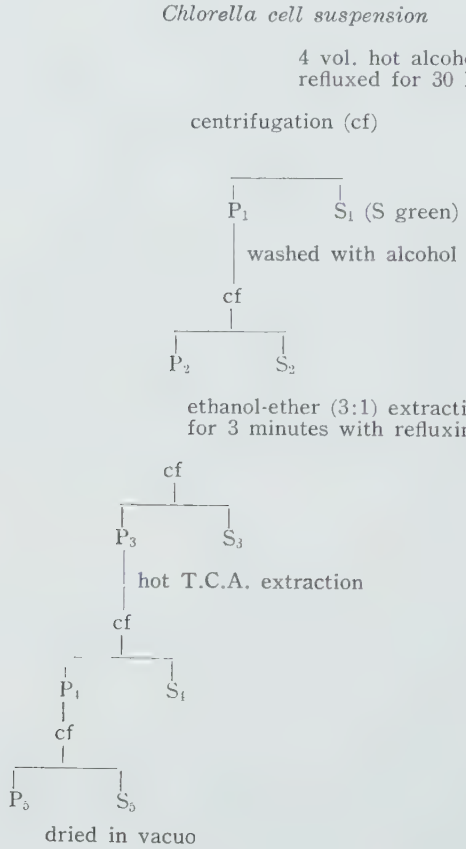
The fraction was acidified with hydrochloric acid, the final concentration of the acid being one normal, extracted with ether in order to remove trichloracetic acid contamination which entered in the preceding process, and heated to 100° for one hour. After hydrolysis, the solution was dried over sodium hydroxide in vacuo. The dried matter was dissolved in a small volume of water and the insoluble material (fumin etc.) was removed by centrifugation and washed with water. The supernatant (about 5–7 ml) was used in the following experiment.

3. Paper electrophoresis of the hydrolysates

One tenth ml of the above hydrolysates was placed on a filter paper strip (2 cm in width) and electrophoresed in a medium of 5 % acetic acid for

2 or 4 hours. The current used was 1 mA/cm. The localizations of the active matters were detected by radioautography, being undetectable by the adsorption of ultra violet light because of their small amounts. The radioactivities were observed on the original spot and in the fraction moved to anode. The acidic component was extracted and studied paper electrophoretically but not in detail (photograph 1).

Table 1. Fractionation of algae components



4. Separation by paper chromatography

The acid hydrolysates and the component moved to anode in the electrophoresis were separated by two-dimensional paper chromatography. The solvents used were isopropanol-hydrochloric acid (41 ml of concentrated hydrochloric acid is added to 70 ml of isopropanol and filled up with water to 250 ml) and ammoniacal butanol (5 % of 28 % aqueous ammonia was added to 86 % (vol/vol) aq. *n*-butanol (4). The spots corresponding to the *Rf* values of adenine, guanine and nucleotides were detected in the chromatogram. More-

over, an unidentified sopt was detected near the front of iso-propanol-hydrochloric acid and several strong radioactive spots were detected near the starting point when ammonical butanol was used as solvent (photograph 2).

5. Separation by ion exchange column chromatography

It has been usually reported that the free purine bases are readily formed during acid hydrolysis of both types of nucleic acids, where as the pyrimidine bases remain for the most part as mononucleotides in the case of PNA or as nucleotide diphosphrates in the case of DNA. Accordingly, the author intended to separate roughly the bases from nucleotides followed by the

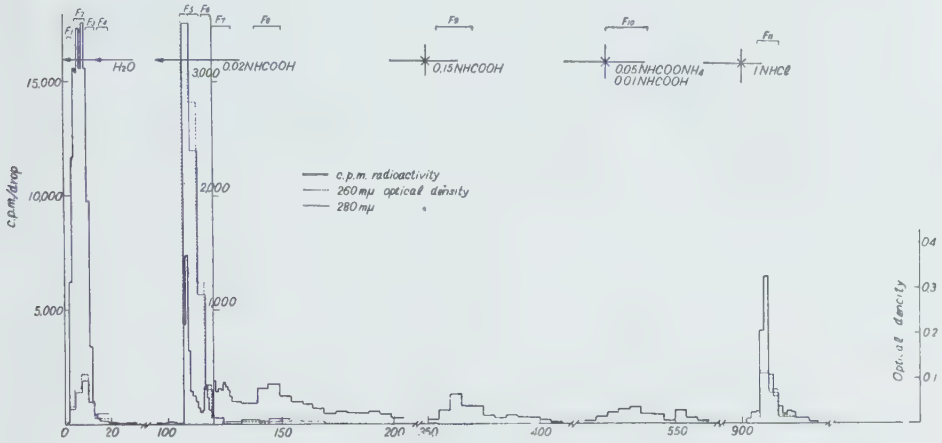


Fig. 1 A. Separation of the purine bases and nucleotides by anion exchange.

Exchanger: Dowex-1-(×8) formate 200-400 mesh 7 cm × 0.64 cm²

Solution: Formic acid, ammonium format as shown.

Sorbed material: Acid hydrohysate of nucleic acid fraction (sample 1) adjasted to pH 8.0 with 3 % NH_4OH.

fractionation of each component. The hydrolysates were neutralized to pH 8 with ammonia, adsorbed on Dowex-1 (formate form) and eluted with formic acid and formic-ammonium formate solution successively. As the first eluates with 0.02 N formic acid contained bases, it was again adsorbed on Dowex-50 (chloride form) and eluated successively with hydrochloric acid.

(a) Preparation of resin column and elution procedure

(1) Separation of nucleotides: Fifteen ml of Dowex-1 (×8, 200–400 mesh) was suspended in water, the fine powder was removed by decantation, put into a glass tube (diameter 0.9 cm) to 7.5 cm, height, washed with 3 N HCl, NaOH and formic acid successively and finally washed 1N formic acid until the absorption of 260–280 mμ of the influent and effluent solution were equal. Then the column was washed with water, and the hydrolyzates run in the column. The flow rate was 0.7–1.0 ml/min. and the effluent was collected every 1–4 ml. The eluting solutions were 0.02, 0.05 and 0.15 N formic acid

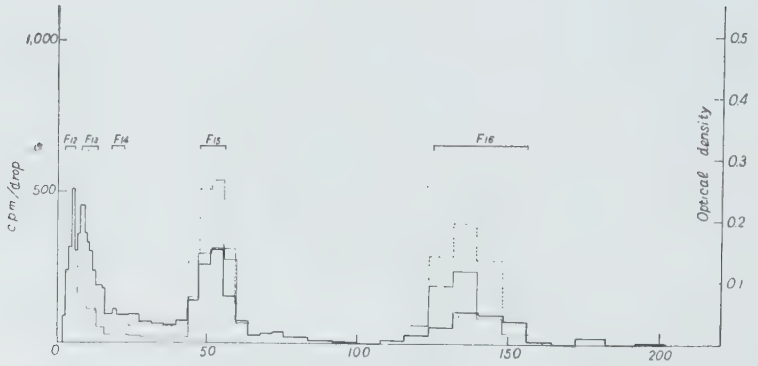


Fig. 1 B. Separation of purine bases by cation exchange in a acid system.

Exchanger: Dowex-50-H+200 mesh 0.64 $cm^2 \times 7.5$ cm
Solution: 2N HCl 1 ml/min
Sorbed material: F5 dried matter in Fig. 1-B in 2 ml 2N-HCl

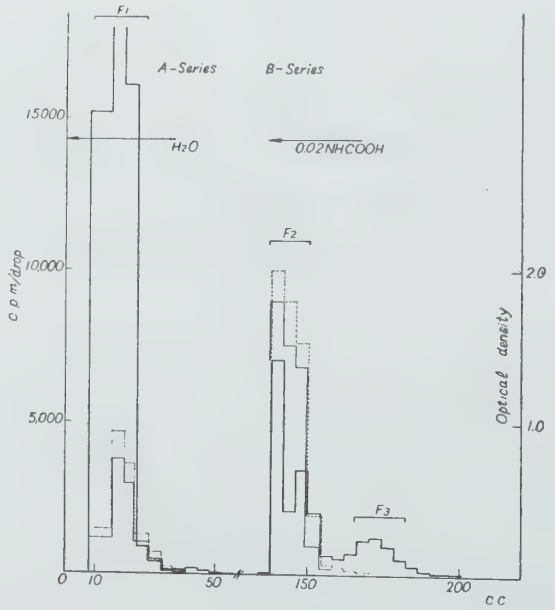


Fig. 2. Separation of the purine bases and nucleotides by anion Exchange.

Exchanger: Same as in Fig. 1-A
Solution: 〃 〃
Sorbed material: Acid hydrolysate of nucleic acid fraction (sample 2)

and mixture of 0.05 N ammonium formate and 0.01 N formic acid successively.

(2) Separation of bases: Dowex-50 (×8, 200 mesh) was put into glass tube (diameter: 0.9 ml) to 7 cm height, washed with 3 N NaOH and 5 N HCl successively and finaly with 3N HCl until the absorption of 260–280 mμ diminished. The fraction which was eluted with 0.02 N formic acid from Dowex-1 column, was collected, dried and dissolved in 5 ml of 2 N HCl and charged on the Dowex-50 column. The column was eluted with 2*n* HCl and the eluate was collected every 4 ml. The flow rate was 0.7–1.0 ml/min.

(b) Measurement of radioactivity and absorption at 260–280 mμ of the effluents

One drop of each effluent was put on a steel dish, dried by infrared lamp and its radioactivity (c.p.m.) was measured by a Geiger-Müller counter. The 260–280 mμ ultraviolet absorption of the high radioactive fractions were measured by a Beckman-type spectrophotometer. The separation diagram obtained from the both measurements was shown in Fig. 1A, 1B and 2.

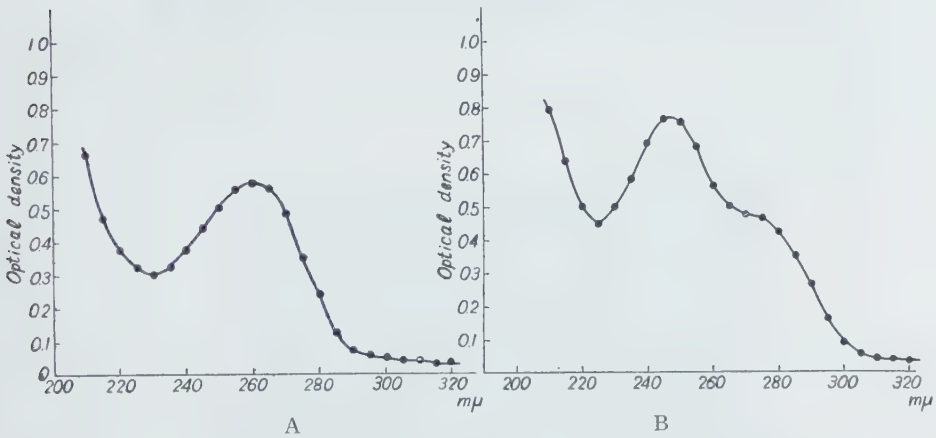


Fig. 3 A. Absorption spectrum of Fraction No. 15 (guanine fraction)
Solution: 0.1 N HCl
Fig. 3 B. Absorption spectrum of Fraction No. 16 (adenine fraction)
Solution: 0.02 N HCl

6. Identification of each fraction

The higher radioactive fraction was dried, dissolved in a small volume of water, subjected to one-dimensional paper chromatography and *Rf* values of active components were determined by radioautography (photograph 3). The ultraviolet absorption spectra of the factions corresponding to adenine and guanine were identified with those of the authentic specimens. (Fig. 3A and 3B)

7. Experimental results

Radioactivity of each fraction obtained by means of the above method was as follows: In experimental, number 1 to 11 fractions were obtained

by Dowex-1 column chromatography. As the total radioactivity was 63.5×10 c.p.m., the process of elution was complete. As No. 5 fraction contained the bases it was chromatographed by Dowex-50 (chloride form), and fractionated into five parts. The sum of activities of fractions No. 15 and No. 16 were adenine (2.2 %) and guanine (1.6 %) respectively. In experiment 2, fraction No. 2 contained a mixture of bases corresponding to 14.6 % of that of the input materials. (table 2)

Table 2. Radioactivity of each fraction

Experiment 1. stating material 63.8×10^5 c.p.m.		Experiment 2 stating material $168.0.6 \times 10^5$ c.p.m.	
Fraction No. 1	7.17×10^5	Fraction No. 1	1250×10^5
2	38.8	2	276
3	5.68	3	53
4	0.525	4	19
5	8.0	5	10
6	1.38	6	4
7	1.01	7	4
8	1.86	8	3
9	0.81	9	10
10	1.54	10	80
11	2.24	11	11
12	0.58		
13	1.06		
14	0.39		
15	1.39		
16	0.71		

DISCUSSION

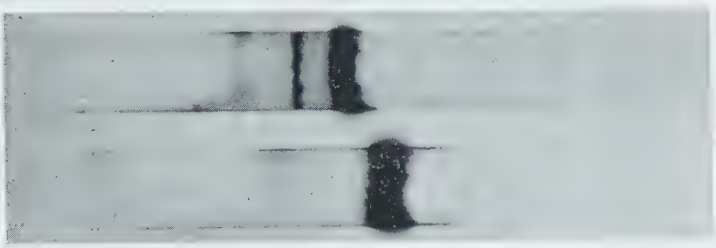
In the above experiments, ^{14}C-labelled adenine and guanine could be separated in fairly pure form from the nucleic acid fraction of chlorella ellipsoidas. The nucleotides could not be isolated in pure state. The material used in this experiment was fractionated by the modified W. C. Schneider's method, i.e., the extraction with cold trichloracetic acid was omitted following the extraction with alcohol. Therefore the nucleic acid fraction was probably contaminated by the many acid soluble substances and the separation thereafter became difficult. It seems to be necessary to remove the acid soluble substances with cold trichloracetid acid or with some other procedures before further treatments.

SUMMARY

Uniformly ^{14}C-labelled adenine and guanine were isolated in fairly pure form from the nucleic acid fraction obtained at the same time when ^{14}C-

Photograph 1.

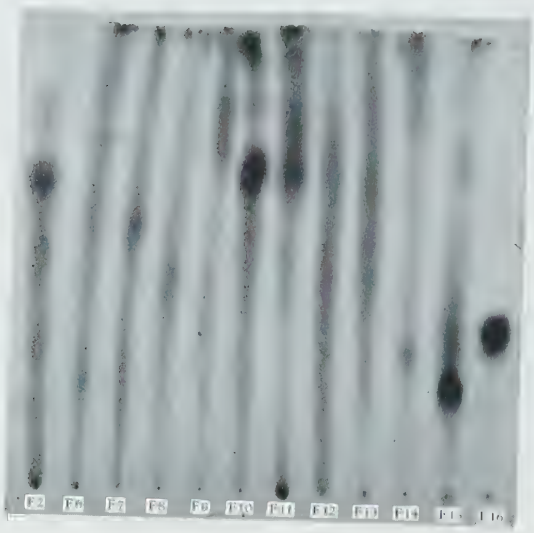
A. The radioautogram of nucleic acid hydrolysate separated by paper electrophoresis.
medium: 5 % $HAc \cdot H_2O$ solution electrophoresed for 2 hours.



B. The radioautogram of acidic fraction of nucleic acid hydrolysate separated by paper electrophoresis.
medium: 5 % $HAC \cdot H_2O$ solution electrophoresed for 4 hours.

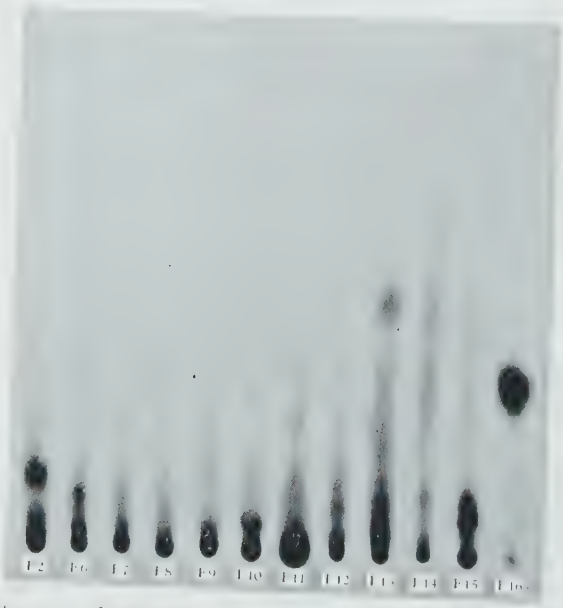
Photograph 2.

The radioautogram of nucleic acid hydrolysate separated by 2 dimensional paper chromatography
solvent: *n*-butanol ammonia and iso-propanal-HCl



Photograph 3.

A. The radioautogram of each fraction of nucleic acid hydrolysate separated by ion exchange resin chromatography
solvent: iso-propanal-HCl



B. The radioautogram of each fraction of nucleic acid hydrolysate separated by ion exchange resin chromatography
solvent: *n*-butanol-HCl

labelled amino acids were isolated from the protein fraction of chlorella ellipsoidae.

ACKNOWLEDGEMENT

The author is indebted to Profs. S. Akabori and B. Maruo for their kind advice in this work.

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STUDIES ON THE MICROORGANISMS FOUND IN THAI RICE AND BURMA RICE

PART 1. ON THE MICROFLORA OF THAI RICE

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FOREWORD

In this paper, the author is reporting the results of microbiological studies of the change of the microflora of the deteriorated rice during his visit to Thailand and Burma for three months, from October 1954 to January 1955, by request of Japanese Government and also some of his later investigation carried out in Tokyo on the samples collected during the trip.

1. *Design and Preparation for Investigation*

With the view of performing ecological observations of microorganisms of the rice as influenced by the factors affecting not only but the production and distribution processes also the change of *Penicillium toxicarium*, *P. citrinum* and *P. islandicum* which are known to produce toxic substances, the author made some arrangment in advance to facilitate quick isolation of microorganisms from collected samples. Equipments and materiales needed for research were shipped by sea to Bangkok and Rangoon in order to make it possible to start the work in time for rice harvest season. The Thailand Government, was kind enough to offer to the author a room in the Laboratory of the Medical Science Bureau, Ministry of Public Health where isolation, culture, microscopic examination of microorganisms and determination of moisture etc. could be performed satisfactority. The only inconveniences were that pertaining to heating for which we had no coal gas at our disposal.

2. *Processes of Production, Treatment and Distribution of Thai Rice*

Before starting with the sampling, of microorganisms the author made a survey on the prevailing conditions involved in production, refining and distribution of Thai rice by investigating informations supplied by the Trade Bureau of the Economic Ministry, Rice Bureau of Ministry of Agriculture, Government of Thailand and also the research data received from the EAC and the FESCO organizations. Here, only a brief outline will be given.

As may be seen from the map in Fig. 1, the fields of rice plantation

are distributed in four main districts. The largest district is the Central Part, the vast paddy fields lying along both banks of the River Menam, producing mostly non-glutionous rice, which is shipped to and from the local centers such as Bangkok, Ayuthaya, Thomburi, Saraburi, etc. The second is the Northeastern Part, extending over a table area, producing both glutinous and non-glutinous rice, which is grown by relatively small farmers. The crop from this part is distributed from towns such as Kohorat, Ubon, Makkhaeng etc. The third is the Northern Part, the well-irrigated paddy field skirting along the mountainous areas, mainly producing glutinous rice which is distributed from towns such as Chiangmai, Chiangrai, Lampang, etc. This part looks like some rice producing areas of Japan. The last and the smallest one is the Southern Part, the rice area along the Northeastern coast of Malay Peninsula. The output from this area is, however, so low that the inhabitants have to depend partly upon imported rice. The Siamese rice, which ranks with the Rangoon and Saigon rice in its history and productivity is largely produced in Parts 1, 2 and 3. Of the total output, the Central part produces 50%, Northeastern 30%, the Northern Part 8%, and the Southern Part only 7%.

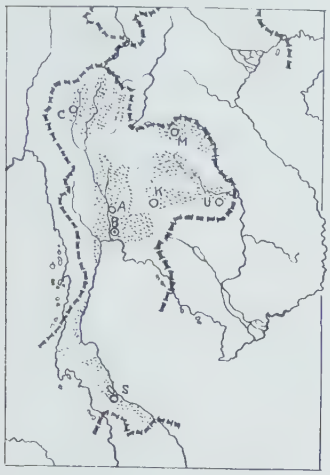

Fig. 1. Rice-producing area of Thailand.

A : Ayuthaya
B : Bangkok
C : Chiangmai
K : Kohorat
M : Makkhaeng
S : Songkhla
U : Ubon

The climate is tropical and although with some slight local difference in rainfall and temperature, the wet season lasts generally from May through October and the arid season from November through March in the following year. On the annual average the temperature in Bangkok is 26–27°C and with a humidity of 80–81%*. With some exceptions according to districts or varieties of rice, seeding or bedding out of seedling are made in the months, June, July and August. The harvest starts at the beginning of November just when the wet season is over and lasts two months. The ways of refining the paddy, that is cutting, drying, thrashing, polishing etc. are still quite primitive. The rice, cut and left in lower stalk about 30 cm above the ground, is packed in bundles and placed on the ground or path in the paddy field for a few days and dried. In submerged paddy fields like those in the Central Part, rice ears often remain reaching below the surface into the water before cutting. Thrashing is done in paddy fields

* Observation values made in 1949~51 in Bangkok—see bulletins published by Thai Navy Meterological Observatory.

after the harvest or in the farm yard by buffaloes turning round and round, tramping upon the rice bundles placed in a circle, 20–30 cm high and afterward grains are collected. This method is used mainly in the Central and Northeastern Parts. In the Northern Part, they hold dried rice bundles with rods and thrash them against a bamboo table or against the inside of a huge bamboo basket about 2.5 meters in diameter and collect grains later. In any case, paddy grains obtained cannot be uniform in dryness. The collected paddy grains are placed in baskets and stored in farm-houses or in a separate storehouse. These paddy store-houses are made of teak with high floors about two meters above the ground and surrounded with verandah, roofed with tiles and with small opening for ventilation, or some are made of coconut leaves and thatched with same.

After reserving some for the farmers' own consumption, all paddy grains are collected and stored in godowns of the local shipping centers through the hands of brokers or cooperative sales corporations. These godowns are made of teak with floor 30–60 cm above the ground and interior space is divided with bamboo walls into some sections, where grains are stored in bulk. Many local godowns have their owe refining mills attached to them. In Thailand, paddy grains are refined by grinding them in a mill into polished rice in one process well free from bran, and the polished rice is packed in gunny bags, 100 kgs net each. About 60% of the total paddy

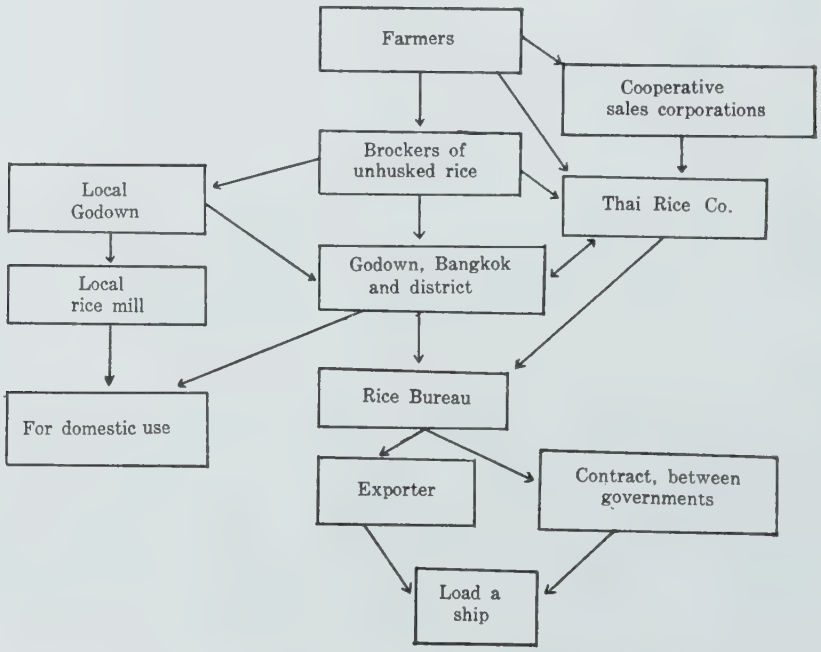

Fig. 2. Distribution Processes of Thai Rice.

crop is refined at producing centers and the balance 40% is sent to refining mills and "Godowns" of Bangkok and paddy districts. Storage of rice, therefore, is largely done in polished rice packed in gunny bags and only a part is stored in bulk in the paddy. In refining new crop paddy of high moisture content in December through February of the following year, it is a usual practice to add old paddy of the previous years crop by 10–30% to facilitate smooth running of grinding mill.

From the chart of Fig. 2, which shows the outline of organization of distribution of rice in Thailand, it may be seen how important a role is played by the dealer called "Godown" who combine wholesale, warehousing and rice refining business in his sole hand. Masters of the Godowns make purchases of polished and unhusked rice from various producing centers, refine them, pack them in gunny bags keep them in stock, and at the request of their buyers, they blend various grades of rice at their own discretion and repack it in gunny bags and ship them to markets. For such blending, various grades of rice such as glutinous and non-glutinous, rice of different crop years, crashed rice is all spread on the floor of the Godown.

Rice for export is shipped from the port of Bangkok and there are about 80 Godowns standing in a long queue along the bank of River Menam, with floors 1–2 meters above water. The typical of these Godowns is about 4 meters high, 10 meters wide in front, and 20–30 meters deep, floored with concrete or thick teak flooring, mostly zinc roofed and wainscoted. It has no air vent in the floor and a very few only in the roof. Looking inside, beams and walls are badly cobwebbed and the floor is infested with cockroaches. The majority of these Godowns is owned by chinese merchants. Nearly two thirds of the total export of rice, amounting to three million tons in the year of the author's investigation, were in the hands of the three big chinese companies, and the balance, about one third, was handled by the Government sponsored Thai Rice Co. This company has very good facilities including the only one mechanized silo in this country and its No. 2 refining mill with processing capacity of 450 tons paddy daily and also its No. 1 warehouse with storage capacity of 6,000 tons polished rice.

Thus, the rice for export is sent by producer, paddy broker and cooperative corporation mostly in the form of polished rice and some in paddy to Bangkok by water or by land and then loaded on board ocean-going boats after going through such processes as refining, blending, packing etc. as described above. In days before World War II, about 60% of total shipment to Bangkok was made in paddy but according to the recent statistics the percentage was reduced to only 40% owing to the increasing percentage of paddy being refined at rice production centers. The refining mills around Bangkok are working only 10 days a month.

3. *Sampling of Specimens and Isolation of Microorganisms*

In order to collect necessary specimens, the author visited many places of the above mentioned four rice producing areas and collected soil, water,

air, rice ears, straw, paddy etc. in the paddy field and the surrounding ground, and collected paddy, polished rice, husks, dust, chip of wall, air, soil of farm yard, etc. at farm-house, Godown, and refining mill.

Sampling of polished rice specimen from the stock stored in Godown was done in the following way: The rice is stored in the Godown, packed in gunny bags each containing 100 kgs in separate lots according to the grade, each in a pile of about twenty bags. From one thousand to five thousand bags of the same grade rice were selected as test lots. From each lot, fifty bags were drawn from top and side and from these bags the author drew samples and mixed them well to make a test sample which was subjected to culture test. Even when the number of bags in one lot was less than 1,000, samples were drawn from fifty bags.

About twenty grams of rice taken from 500 to 1,000 g of rice samples at random in sterilized Erlenmeyer flask, 250 cc in capacity, and washed by shaking it 20 times with each about 100 cc sterilized water. This is for the reason of washing away the fungus spores attaching to the grains. These operations should be done in a germ-free case. After repeating the above washing for twenty times, the kernels were placed one by one on the Czapek agar medium in each test-tube up to 100 or 200 kernels and held for 7 to 12 days at 28°±2°C and then examined the species and number of microorganisms developed on the kernels. In other test samples, microorganisms were isolated by dilution method with Czapek agar and boullion agar medium and for air plating culture in Petri dish was used. On wash-fluid, isolation tests were made on wash-fluid of the first washing and that of the twentieth washing. Because isolating conditions and considered of perticular importance for this ecological investigation, isolation tests were made on the whole test samples upon returning to Tokyo in the following method: Polished rice kernels and paddy kernels and their husked rice kernels are placed in a shaker flask and after adding 150 cc sterilized water and washed by sufficiently shaking to make the first wash-fluid. Then the sample was given two washing by shaking in 150 cc of 0.01% Tween 80 solution and eight more washings in 150 cc sterilized water to make the last wash-fluid. After last washing, the sample was placed on three different agar media, Czapek, Koji extract and peptone glucose spread on Petri dish, by placing five sample kernels on each plate up to 100 kernels about each media and isolation was done by breeding at 28° and 37°C. At the same time, the first and last wash-fluids were subjected to isolation testing by means of plate culture at such dilutions as 10^{-3}, 10^{-4} and 10^{-5} respectively under the same conditions as described above.

EXPERIMENTAL RESULTS

The results of the above tests are given in the varieties of the microorganisms and the number of their colonies found in various specimens as

you will see noted in Table 1 through Table 8 in case of Czapek medium at 28°. In these tables, P.i. indicates *Penicillium islandicum*, P.c.=*P. citrinum*, O.P.=Other Penicillia, A=*Aspergillus*, (W.A.)=the number of white *Aspergillus* in *Aspergillus*, O.F.=Other moulds than *Penicillium* and *Aspergillus*, St.=*Streptomyces*, B=bacteria, Y=yeast, N=number of kernels not infested with internal microflora per 100 kernels.

The number of colonies of *P. islandicum* and *P. citrinum* are shown under separate items because these are specified by the Food Board and Ministry of Public Health of Japan as harmful moulds infesting the so-called deteriorated rice together with *P. toxicarium* and the subjects of their inspection. Because *P. toxicarium* was not isolated from any of the specimen during present tests, these tables have no item special for this mould. Of *Aspergillus*, the number of white *Aspergillus* is given separately because the number was characteristic and the mycological study on this moulds will be reported in a separate paper later[4]. The data on the species of other microorganisms such as O.P., A., O.F., St., B, Y is too extensive to be listed in the tables in the present paper. But, these data, being highly important to the study of the change of microflora, will be outlined in explanation of experimental result. In description of specimen, GD is for Godown, RM for refining mill, and % indicates contents of crashed rice.

1. *Microflora of Old Crop Rice* (*see Table 1*)

Here the old crop rice denotes rice of 1953–4 crop or about one year

Table 1. Microflora of old crop rice.

Samples \ Microflora		Pi	Pc	OP	A	(WA)	OF	St	B	Y	N
Bangkok area	Eah Thai GD-No. 1, 10%	4	0	7	18	16	13	5	39	0	29
	Thai Sae GD, 15%	4	1	5	12	9	18	4	29	0	33
	Siang Hua Long RM, 10%	0	0	3	29	27	7	5	13	0	45
	Wang Lee RM, 15%	1	0	4	57	40	2	1	21	0	17
	Sithiphol GD, 5%	0	2	5	44	32	16	20	8	0	8
	Hon Yae Seng RM,	1	0	8	25	20	1	9	14	0	46
	Taharn GD-No. 1, 10% (glutinous rice)	0	1	9	71	67	6	0	5	0	18
Ayuthaya area	Thai Mongknol RM, 10%	0	4	13	21	18	2	0	21	0	38
	Nguan Hoat Sen RM, 10%	1	0	6	5	4	8	4	40	0	37
Sara-Buri area	An Fong Lao GD, 10%	2	1	2	26	22	5	7	28	0	40
Chiangmai area	Min Ko RM*	0	0	0	0	0	1	0	14	0	85
	Sangpatong RM* (glutinous rice)	0	0	3	2	0	3	0	13	1	87

* Freshly-polished old crop rice.

old from the time of the harvest and stored as polished rice. As shown in Table 1, *Aspergillus* predominates among moulds and the majority consists of white *Aspergillus*. In yeast, *Rhodotorula* was only rarely found even when isolation was made in the Koji extract media. The majority of bacteria was of the *Bacillus megatherium*. Little attention had been paid to *Streptomyces* in rice before, but in the present investigation, this was found in many specimens and when isolation is done in the media after adding fungicides such as Eurocidin or Kabicidin almost each old polished rice kernel will develope on its surface a considerable number of its colonies. And it was found that these *Streptomyces* mostly are consisting of *St. diastaticus* like strains and are considerably poisonous to nervous system. When mice were fed with food containing 10% of rice with these strains grown on it, the symptoms of disease appeared with in several days and all of them died during the following several days. In old polished rice, the number of non-infested kernels was generally from 10 to 50% of total number of the kernels. In contrast with kernels stored in the from of polished rice, two examples are shown in the bottom line marked * in Table 1, showing a part of the data obtained from the old rice kernels, immediately after polishing, from those stored in paddy. In this case, the number of non-infested kernels predominated, exceeding 80% and the number of moulds, even *Aspergillus*, was very small. From these experimental results, rice will be stored in paddy (unhusked grains), which affords much greater safety than in polished form in Thailand.

2. *Microflora of New Crop Rice (see Table 2)*

This new crop rice indicates 1954–55 crop rice, about one month old from the time of the harvest and about one to two weeks after refining. In experimental result, the new crop rice in general (marked * in Table 2) showed no wide difference compared with the case of the old polished rice. In the freshly polished new crop rice sampled at refining mill (marked ** in Table 2), a great difference was recognized from the case of freshly polished old crop rice (shown in the bottom line in Table 1), the former giving a smaller number of non-infected kernels but a markedly high number of bacteria, about 60–80, mostly comprising Yellow *Pseudomonas*, while the latter gave only from 10 to 20 of bacteria. As to moulds found in freshly polished new crop rice, very few *Aspergillus* and *Penicillium*, and instead rather abundant Mucorales, *Alternaria*, *Curvularia*, *Cepharosporium*, etc. were detected.

In Table 2, the specimen marked *** represents the type prepared by blending the freshly-polished new rice with rice of other grades in blending operation, one of the preparation processes unique in Thailand. In the experimental result, this specimen gave 60–80 bacteria, mostly Yellow *Pseudomonas*, and also fairly many *Aspergillus*, predominated by white *Aspergillus*, *Penicillium*, and other moulds. And from the new crop rice kernel some time old after polishing fairly, many *Streptomyces* were detected.

Table 2. Microflora of new crop rice.

Samples \ Microflora		Pi	Pc	OP	A	(WA)	OF	St	B	Y	N
Bangkok area	Eah Thai GD	0	1	0	56	48	5	2	16	0	23*
	Soon Heng Lee GD	1	1	0	64	56	4	3	23	0	8*
	Wang Lee GD-No. 1	0	1	2	21	18	13	4	35	0	29*
	Wang Lee GD-No. 2	1	0	2	28	25	13	6	28	0	29*
	Long Heng Chan RM, 5%	0	0	3	52	49	3	2	24	0	23*
Kohoraht area	Kohoraht RM	0	0	1	32	—	3	5	23	0	43*
Chiang-mai area	Genhatsuten RM	0	0	1	0	0	31	0	76	0	12**
Bangkok area	Hoi Sae RM	0	1	1	0	0	0	0	46	0	34**
	Chin Seng Lee RM	2	0	5	81	72	4	0	15	0	0****
	Hua Heng Chan RM, 5%	1	0	3	37	32	3	0	73	0	0***

* New crop rice.
** Freshly-polished new crop rice.
*** After blending freshly-polished new rice with the other grades.
**** Glutinous rice, produced in Northern part.

From the preceeding facts, it is supposed that a significant change of microflora would take place from immediately after to some time after polishing and even new crop polished rice would give, some time after polishing, trend of movement of microflora similar to that of the old crop polished rice. In the glutinous rice produced in the Northern Part (marked **** in Table 2), particularly many white *Aspergillus* are frequently detected from new crop polished rice and such fact was found true also in Burma with the new crop polished glutinous rice[1].

3. *Microflora of Old Crop Unhusked Rice* (*see Table 3*)

In Thailand rice is usually stored in the form of polished rice but even at the time when the new harvest season begins there is the previous years crop rice stored in paddy in storehouses in producing areas. Therefore, the experiments were made on this paddy rice in the same method as used for polished rice and the results are shown in Table 3. In number of microorganisms *Aspergillus* comes to the top, followed by *Penicillium* and Mucorales. In the item O.F. in Table 3, 60–95% are represented by Mucorales. In these specimens, *Aspergillus* is rich in species with Yellow-Green *Asp.* predominating with 10–60%, followed by Black *Asp.*, representing 0.5–30%, Brown *Asp.*, representing 5–25%, Yellow *Asp.*, 5–15%, *Asp. glaucus* group under 8%, *Asp. terreus* group 5–10%, White *Asp.* 3–8%, *Asp. fumigatus* group, *Asp. nidulans* group and *Asp. versicolor* group, each representing less than 5%. These species or varieties of *Aspergillus* have been found

Table 3. Microflora of old crop unhusked rice.

	Samples \ Microflora	Pi	Pc	OP	A (WA)	OF	St	B	Y	N
Bangkok area	Thai Sae RM	—	—	14	83	0	17	0	3	0	0
	Hua Heng Chan RM, 17	—	—	3	69	0	24	0	6	0	0
	〃 15	1	1	6	71	3	28	0	5	0	2
	〃 26	0	1	4	98	5	52	0	—	0	2
	〃 21	1	1	2	28	1	26	0	38	0	0
	〃 24	1	1	9	38	2	13	0	40	0	0
Banpain area	Banpain farm-house No. 35	7	9	9	63	0	19	0	6	0	0
	〃 No. 2	4	14	5	51	0	23	0	2	0	0
Ayuthaya area	Ayuthaya farm-house No. 14	0	5	17	34	5	44	0	12	0	0

to be similar in trend to the result of isolation made later with Koji-extract agar media. In this experiment on unhusked rice, almost all kernels showed germination, although not same period of time was required. Next, experiments were made in the same method by washings and isolation in culture media on an unhusked but unpolished rice kernel still covered with pericarp which is called hulled rice or brown rice. The experimental result showed a significant decrease in the number of moulds down to 20–50% of total found in paddy. And number of bacteria was 20–60 per 100 kernels of hulled rice, but 0–40 per 100 kernels of unhusked rice. And of the species of these bacteria Yellow *Pseudomonas* predominated and *Bacillus* was relatively less. Rice kernels in these form of the hulled rice showed active germination. And by viewing the relation of such germination and microorganisms, it was found that higher percentage of germination was seen in the specimens containing Yellow *Pseudomonas* higher in number than that of other bacteria. This was also found to be true in the case of paddy rice. The result of our investigation on such corelation has been reported in details in the separate article together with the experimental results on Japan unhusked rice found later by the author[2].

4. *Microflora of New Crop Unhusked Rice* (*see Table 4*)

The new crop unhusked rice specimen kernels collected from rice ears immediately before or after harvesting or from rice bundle during sun-drying, thrashing, and storing processes, were hand-unhusked with a disinfected pincette and separated into the husked rice kernels and husks, each of which were subjected to the isolation tests in the same method as described above. From the results of this test, the representative examples are shown in Table 4. In this instance, the husked rice gives very few *Aspergillus* and *Penicillium* in moulds majority of which consisting of *Alternaria*, Mucorales, *Curvularia*, *Cepharosporium*, *Helmintosporium* and other

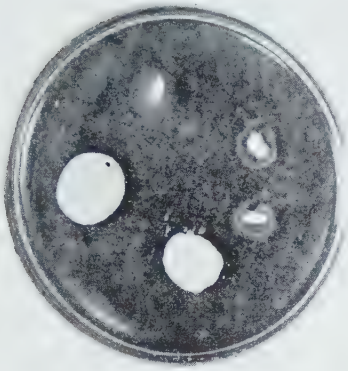

Fig. 3. The old crop rice kernels plated on Czapek ager at 28°, and showing colonies of *Bacillus megatherium* (B) (C) and other *Bacillus* sp. (D) (E) and White *Aspergiltus* (A) growing out. (see Table 1).

Fig. 4. The new crop rice kernels, non-freshly polished, plated on peptone glucose agar at 28°, and showing colonies of *Pseudomonas lacunogenes* (A), *Bacillus* sp. (E), White Aspergillus (B), *Penicillum* sp. (C) and imperfect fungi (C) growing out and non-infested kernel (D). (see Table 2).

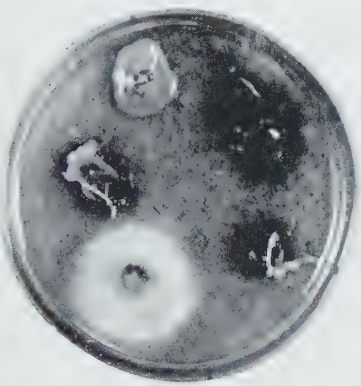

Fig. 5. The new crop husked rice kernels plated on potato agar at 28°, and showing germination of the seeds and colonies of Yellow *Pseudomonas* (A) (B) (D) (E) and imperfect fungi (B) (C) (D) (E) growing out. (see Table 4).

Fig. 6. The new crop husked rice kernels plated on potato ager at 28°, and showing germination of the seeds and colonies of Yellow *Pseudomonas* (A) (B) (C) (D) (E) growing out. (see Table 5).

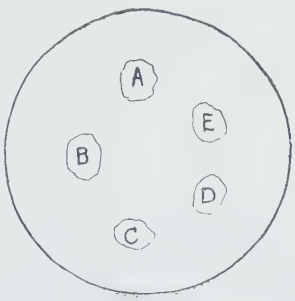

Table 4. Microflora of new unhusked rice.

	Samples \ Microflora	Pi	Pc	OP	A	OF	St	B	Y	N	
Sangpatong area	Matured glutinous rice ears	0	0	1	2	95	0	66	0	0	husked rice
		1	0	3	1	100	0	4	0	0	husk
	Glutinous rice after thrashing	0	1	0	2	81	0	68	0	0	husked rice
		1	4	6	1	102	0	22	0	0	husk
Saigenhatsu RM in Chiangmai area	Non-glutinous rice in trade	0	0	0	0	100	0	68	0	0	husked rice
		0	1	0	4	104	0	10	0	0	husk
	Non-glutinous rice in paddy godown of rice mill	0	0	0	2	92	0	46	0	0	husked rice
		0	0	3	1	100	0	1	0	0	husk

non-sporeforming moulds. A fairly large number of bacteria was detected and the majority of it was Yellow *Pseudomonas*.

5. *The Change of Microflora found in Old and New Unhusked Rice During Refining Process* (*see Table 5 and 6*)

In the refining mills in Thailand, unhusked rise is refined by grinding into highly-polished rice in one process, during which ample bran contents are removed from the grains. For the purpose of our investigation, old crop kept in storage for past twelve months and new crop, about twenty days old from of time the harvesting in Chiangmai area, were both sent to

Table 5. The change of microflora in old unhusked rice during refining process.*

Samples \ Microflora	Pi	Pc	OP	A	(WA)	OF	St	B	Y	N
Unhusked rice in trade	0	0	2	86	0	22	0	0	0	0
Sorted unhusked rice	9	1	6	64	0	16	0	10	0	0
Half husked rice	22	5	21	30	8	13	2	24	0	1
Husked rice	6	0	14	30	20	17	0	50	0	0
Half polished rice	0	0	4	52	44	14	6	28	0	14
Polished rice	0	0	8	10	8	4	12	48	0	28
Crashed rice	0	0	0	2	2	8	0	16	0	74
Bran	1	0	0	7	5	many	0	many	0	—

* Harvesting in Dec. 1953, refining tests in 18th Nov. 1954 at Saigenhatsu rice mill, Chiangmai area,

Table 6. The change of microflora in new unhusked rice during refining process.*

Microflora / Samples	Pi	Pc	OP	A	(WA)	OF	St	B	Y	N
Unhusked rice in trade	0	0	0	1	0	78	0	83	0	0
Sorted unkused rice	0	0	0	2	0	71	0	67	0	0
Half husked rice	0	0	0	0	0	79	0	81	0	0
Husked rice	1	1	1	1	0	76	0	77	0	1
Half polished rice	0	0	0	1	0	32	0	92	0	1
Polished rice	0	0	0	0	0	28	0	91	0	1
Crashed rice	0	1	0	1	0	33	0	91	0	4
Bran	0	0	0	0	0	many	0	many	0	—

* Harvesting in Oct. 1954, refining tests in 19th Nov. 1954 at Taihoyu rice mill, Chiangmai area.

the refining mill for grinding and specimens were collected at every stage of the refining process. These specimens were subjected to test about forty hours later. The results obtained with old crop are given in Table 5 and the results with new crop are listed in Table 6. The results of these experiments are summarized as follows: (1) In new crop, both sorted and unsorted unhusked rice, more O.F. and B. were detected, while in old crop marked less O.F. and B. but more *Aspergillus* and *Penicillium* were found. This tendency becomes more apparent in the stage of the hulled rice. (2) In the stage of polished rice the trend as given in (1) still continues, but in the polished rice newly refined from new crop, *Aspergillus*, *Penicillium* and *Streptomyces* were found to be very few but a large number of bacteria, predominated by Yellow *Pseudomonas*, were detected. These Yellow *Pseudomonas* is also found in newly polished rice from old crop. (3) In the present experimental method, larger number of non-infected kernels were found in the rice refined from the old crop than in the rice from new crop. (4) From the newly refined rice of the old crop, fairly many *Streptomyces* were found.

In refining new crop by grinding during the period from December through March the following year, old crop is usually added before grinding in proportion of 10–30% to the total to reduce average moisture of paddy grains. On studying change of microflora, the influences from those factors must be taken into account.

6. *Microflora in the Environments relating to Preparation Processes of Rice* (*see Table 7*)

Samplings were made for various specimens from the environment involved in preparation and distribution of Thai rice in order to study on the movement of microflora by dilution method at 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. As noted in Table 7, distribution of the microorganisms detected from polished

Table 7. Microflora in the enviroments relating to preparation processes of rice.

Microflora / Samples	Pi	Pc	Pt	OP	A	(WA)	OF	St	Y+B	N	dilution
Dust beside rice polisher	0	0	0	17	25	0	41	0	many	—	10^{-3}
Dust in paddy godown	0	4	0	135	23	0	85	0	many	—	10^{-3}
Dust on old paddy	2	9	0	0	9	0	many	0	many	—	10^{-3}
Soil of paddy field A	1	0	0	4	9	0	4	3	many	—	10^{-4}
〃 B	0	0	0	6	4	0	5	2	14	—	10^{-4}
〃 C	0	1	0	3	0	0	many	1	5	—	10^{-4}
Soil of road	0	0	0	0	0	0	1	0	0	—	10^{-4}
Damaged rice on floor of Eah Thai GD	6	2	0	22	56	5	26	12	12	1	- -

rice or unhusked rice is quite extensive. From the rice specimen, possessing visible deterioration, collected from hollows in Godown floor, particularly many *Aspergillus*, *Penicillium* and *Streptomyces* were usually isolated. Yellow *Pseudomonas*, abovementioned, are often isolated from the wet paddy field soil.

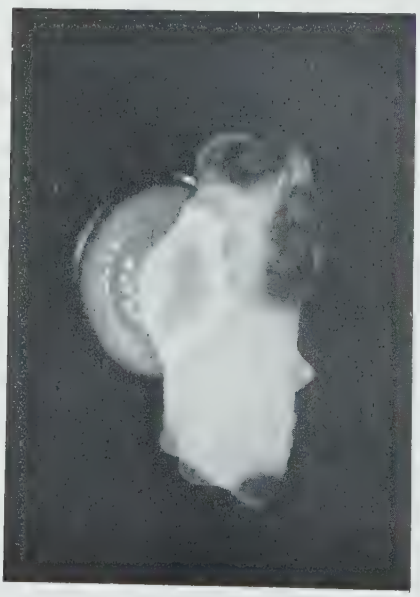

Fig. 7. The old crop rice kernel plated on Gzapek ager at 28°, and showing colonies growing out.

7. *Microflora of Rice for Local Consumption* (*see Table 8*)

To know about microflora of the rice daily consumed by Siamese people,

Table 8. Microflora of rice for local consumption.

	Microflora / Samples	Pi	Pc	OP	A	(WA)	OF	St	B	Y	N
Chiangmai area	Farm-house for domestic use, mixed with black rice, fresh-polishing new rice	0	0	0	0	0	25	0	75	0	11
	Taihoyu RM, fresh-polishing new rice	0	1	0	0	0	27	0	88	0	1
	Sangpaton rice shop, old glutinous rice	0	0	2	27	—	1	1	17	0	43
	Sangpaton rice shop, old non-glutinous rice	0	4	16	24	—	8	0	22	0	26
Kohoraht area	Karaku rice shop	0	0	1	32	—	3	5	23	0	43
Songkhla area	Hatyai, special class	4	1	29	24	21	2	0	5	0	39
	Hatyai, 1st class	2	0	26	56	45	7	6	19	0	1
	Hatyai, 2nd class	4	2	24	27	19	19	3	13	0	0
	Songkhla farm house, old rice for domestic use	0	1	0	53	—	19	31	60	0	10
	Nakane RM old rice	0	0	37	26	—	8	2	18	0	18
Bangkok area	Eah Thai GD	1	1	2	18	6	6	1	40	0	31

specimens were collected from stocks of retailers and farmhouse stores and tested. The results are partly showen in Table 8. Due to difficulties from characteristic distribution system in Thailand and from the authors lack of command on Siamese language, adequate informations as to crop years, places of production, ways of storage, etc., were not obtainable. Therefore, these experimental results cannot be used as basis for further conclusion. In the Par-boiled rice, a special type of rice with some peculiar odor, prepared by boiling and drying in unhusked grains and then refined by grinding and known to give smaller crashed rice during grinding, no facts were found to justify popular belief that this type of rice is less liable to contract microbiological pollution and from some kernels of this type were found to contain fairly many *Penicillium*, as much as 10–20 colonies in 100 kernels.

SUMMARY

In investigating the causes of deterioration of cereal grains under the influences from microorganisms, the author has been of the opinion that the most important approach is to trace the movements of microflora in an ecological system termed cereal grains. This microbiological studies on the cause of the deteriorated rice, have been carried out in the same light. The

results of this investigation are summarized as follows.

(1) The significant change of microflora after the normal maturity, would take place from immediately after to some time after polishing. In the freshly-polished new rice kernel after removing husks (inner- and outer-glume), bran layer (pericarp, endosperm,) and sometime a part of aleuron layer, the microorganisms show significant decrease in number and Yellow *Pseudomonas* and some moulds known to invade rice during cultivation, such as *Alternaria*, *Curvularia*, *Helmintosporium*, *Cepharosporium*, etc. are found still remaining. However, soon after the polished rice is put in storage, the number of these microorganisms rapidly increase and instead come moulds such as White *Aspergillus*, bacteria such as *Bacillus megatherium* and *Streptomyces* begin to predominate.

(2) In normal rice which passed the wet season in storage from of polished rice, fairly many moulds such as *Aspergillus* mostly consisting of White *Aspergillus* and *Penicillium* are found and not much change is seen in bacteria in which *Bacillus megatherium* still predominates. But, it is a noteworthy fact that particularly many *Streptomyces* such as *Streptomyces diastaticus*, etc. are found in this specimen.

(3) In the polished rice after passing the wet season in storage, there is some rice of which deterioration can be recognized by visual inspection, for example FESCO standard. The common feature of these deteriorated rice is that *Aspergillus*, *Penicillium* and *Streptomyces* are found to be considerably more than in the normal rice. Therefore, deterioration of rice caused by microorganisms is considered to take place in Thailand depending upon the extent of increase of *Aspergillus*, *Penicillium* and *Streptomyces* during the storage period of polished rice.

(4) No attention has been paid to *Streptomyces* found in rice up to the present day. But, many of these were found not only in almost every specimen of normal polished rice but also in freshly-polished rice from the unhusked rice after one years storage. And these *Streptomyces* of rice mainly consist of *Streptomyces diastaticus* like strains. And it was found that some of these gaves considerably poisonous function when mice were fed with food containing rice with the strain grown on it.

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